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EFFECTS OF SELECTED ORGANIC DRINKING WATER CONTAMINANTS ON MALE REPRODUCTION

by

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16. ABSTRACT

Because of the recent increase in exposure of individuals to potentially harmful chemicals, it has become increasingly important to test the potential of environmental chemicals to cause adverse reproductive effects. The Division of Toxicology within the Department of Pharmacology, Medical College of Virginia has responded to this need by examining the abilities of Kepone®, hexachlorobenzene, 2,4-dinitrotoluene, 1,2,3,4-tetrabromobutane, chloral hydrate, 1,1,2-trichloroethylene, 1,2-dichloroethylene, 1,2-dichloroethane, dibromochloromethane, trichloromethane, and 1,1,1-trichloroethane to elicit harmful reproductive effects. The following tests were used to assess the extent of these effects: analysis of effects upon rat ejaculate volume and sperm morphology, distribution studies in rats, determination of the cellular sites of action in a P388D_1 lymphoid neoplasm cell line, performance of a multigeneration murine experiment which included dominant lethal and teratologic studies, and analysis of the inhibition of mouse testicular DNA synthesis.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of the environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicology, developmental and experimental biology and clinical studies using human volunteer subjects. These studies address problems in air pollution, water pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides and other chemical pollutants. The Laboratory participates in and provides data for the development and revision of criteria documents on pollutants for which national ambient air quality and water quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U. S. inhabitants.

In recent years, there has been an increase in the environmental exposure of individuals to compounds having the capacity to cause adverse reproductive effects. A means of identifying these compounds must be developed in order to determine which compounds should be restricted in our environment. The Division of Toxicology within the Pharmacology Department of the Medical College of Virginia - Virginia Commonwealth University is assisting in this endeavor by assessing the potential of organic contaminants found in drinking water to induce adverse reproductive effects. The results of tests evaluating the potential reproductive effects of chlordecone (Kepone®), hexachlorobenzene, 2,4-dinitrotoluene, 1,2,3,4-tetrabromobutane, dibromochloromethane, trichloromethane, 1,1,1-trichloroethane, chloral hydrate, 1,1,2-trichloroethylene, 1,2-dichloroethylene, and 1,2-dichloroethane in rats or mice are presented in this report. Conclusions have been drawn concerning the capacities of these compunds to induce adverse reproductive effects. Recommendations for subsequent experimentation have also been included.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

This research project was initiated with the overall objective of determining the capabilities of the compounds tested to induce adverse reproductive effects. In order to fulfill these objectives, several tests using either mice or rats were employed.

Distribution studies of ^{14}C labelled dinitrotoluene and Kepone® were performed. The amount of compound per gram of various body fluids and tissues was calculated at time points ranging from 2 hours to 84 days post-exposure.

Chloral hydrate, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, and 1,1,2-trichloroethylene were tested for their effects upon mouse testicular DNA synthesis. The extent to which each compound was capable of inhibiting DNA synthesis was calculated, and the data were analyzed statistically. Comparisons of the effects of these structurally similar compounds were also made.

The effects of Kepone® and hexachlorobenzene on the volume of ejaculate and the number of abnormal sperm in the rat were determined.

Using a $P388D_1$ cell line, the cellular sites of action of Kepone® were determined. Growth studies, phagocytosis of opsonized sheep red blood cells, cellular respiration, binding of Kepone®, and calcium efflux were measured at different concentrations of administered Kepone®.

Dominant lethal effects induced by dinitrotoluene, tetrabromobutane, Kepone®, and hexachlorobenzene were studied. Several indices (i.e. mating, fertility, preimplantation loss) were calculated and analyzed statistically for deviations from vehicle control values.

Dibromochloromethane and trichloromethane were used in a modified multigeneration murine reproduction study. 21-day survival studies were conducted on all litters. Adult weight gain and fluid consumption were continuously monitored, and all animals were necropsied to determine target-organ effects. The study also included the analyses of dominant lethal and teratogenic effects. All data were analyzed statistically for significant deviations from vehicle control values.

This report was submitted in fulfillment of Grant No. 804290010 by Drs. J.F. Borzelleca and R.A. Carchman under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period March 1, 1976, to December 31, 1979, and work was completed as of January 31, 1980.

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CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD; BEGIN SECTIONS HERE

CONTENTS

Foreword . . . iii
Abstract . . . iv
Figures . . . vii
Tables . . . xi
Abbreviations and Symbols . . . xii
Acknowledgment . . . xiii
1. Introduction . . . 1

2. Conclusions

2,4-Dinitrotoluene (DNT) . . . 4
1,2,3,4-Tetrabromobutane (TBB) . . . 4
Kepone® . . . 4
Hexachlorobenzene (HCB) . . . 5
Dibromochloromethane (DBCM) and Trichloromethane (TCM) . . . 5

Inhibition of Testicular DNA Synthesis

Chloral Hydrate (CHL) . . . 5
1,1,2-Trichloroethylene (TCE 2) . . . 5
1,1,2-Trichloroethane (TCE 1,2) . . . 5
1,1,1-Trichloroethane (TCE 1,1) . . . 5
1,2-Dichloroethane (DCE 1,2) . . . 5
Compound Comparisons . . . 5
3. Recommendations . . . 7

4. Materials and Methods

Test Strains . . . 9
Materials . . . 9

5. Experimental Procedures

Dominant Lethal Studies . . . 13
Collection of Ejaculate in the Rat . . . 13
Analysis of Ejaculate and Examination of Sperm Morphology . . . 14
Distribution Studies . . . 14

Cellular Sites of Action

Methodology . . . 14
Growth Studies . . . 15
Phagocytic Assay . . . 15
Cellular Respiration . . . 15
Cellular Binding . . . 16
Calcium Efflux . . . 16

Modified Multigeneration Reproduction Study

Animal Husbandry . . . 16
Drinking Solutions . . . 17
Experimental Design . . . 17
Observations . . . 17
Dominant Lethal Screening . . . 18

BEGIN LAST LINE OF TEXT
BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS
3/8"
PAGE NUMBER
EPA Form 2350-4 (4-80)
(PREVIOUSLY CIN. EPA FORM 287)
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TYPING GUIDE SHEET

Teratology Screening . . . 20
Necropsy . . . 20
Inhibition of Testicular DNA Synthesis . . . 20

6. Results and Discussion

2,4-Dinitrotoluene (DNT)

Dominant Lethal Study . . . 24

1,2,3,4-Tetrabromobutane (TBB)

Dominant Lethal Study . . . 33

Kepone®

Dominant Lethal Study . . . 38
Distribution Study . . . 43
Effects on Sperm Morphology . . . 45
Cellular Sites of Action . . . 45

Hexachlorobenzene (HCB)

Dominant Lethal Study . . . 58
Effects on Sperm Morphology . . . 64

Dibromochloromethane (DBCM) and Trichloromethane (TCM)

Modified Multigeneration Reproduction Study

General Observations . . . 64
Fertility . . . 64
Litter Size at Birth . . . 73
Gestation Survival . . . 73
Postnatal Survival . . . 73
Postnatal Body Weight . . . 73
Necropsy . . . 77
Dominant Lethal Screening . . . 77
Teratology Screening . . . 77

Inhibition of Testicular DNA Synthesis

Positive Controls

Methylmethanesulfonate (MMS) . . . 89
Daunorubicin (DNR) . . . 89

Experimentals

Chloral hydrate (CHL) . . . 98
1,1,2-Trichloroethylene (TCE 2) . . . 98
1,1,2-Trichloroethane (TCE 1,2) . . . 98
1,1,1-Trichloroethane (TCE 1,1) . . . 105
1,2-Dichloroethane (DCE 1,2) . . . 105
Compound Comparisons . . . 105
References . . . 120

BEGIN FIRST LINE OF TEXT HERE
CENTER OF PAGE
TOP OF IMAGE AREA
DROPPED HEAD. BEGIN SECTIONS HERE

FIGURES

Number | Page
1 Structures of the compounds tested 11
2 Flowchart for the modified-multigeneration reproduction study. 19
3 Isolation of mouse testicular DNA. 22
4 Mean body weight changes of male rats after receiving five oral doses of DNT in a dominant lethal study 25
5 Effect of fourteen daily doses of Kepone® on male rat body weight. N=6. Vertical bars indicate SEM. 47
6 Effect of fourteen daily doses of Kepone® on volume of ejaculate in the rat. N=6. Vertical bars indicate SEM. 48
7 Effect of fourteen daily doses of Kepone® on sperm count in the rat. N=6. Vertical bars indicate SEM 49
8 Effect of fourteen daily doses of Kepone® on percent morphologically abnormal sperm in the rat. N=6. Vertical bars indicate SEM. 50
9 Effect of various concentrations of Kepone® and dinitrophenol on the proliferation of P388D$_1$ cells. N = 5. Vertical bars indicate SEM. 51
10 Effect of various concentration of Kepone® and dinitrophenol on oxygen consumption by P388D$_1$ cells. Three minute preincubation period with both agents. N=4. Vertical bars indicate SEM 53
11 Effect of various preincubation periods with $1\mathrm{x}10^{-5}$M Kepone® on oxygen consumption by the P388D$_1$ cells. N=5. Vertical bars indicate SEM. 54

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12 Effect of time on the binding of various concentrations of Kepone® to the P388D$_1$ cells. N=4; mean. 56

13 Effect of time and concentration of Kepone® on the ability of P388D$_1$ cells to phagocytize opsonized sheep red blood cells. N=5. Vertical bars indicate SEM 57

14 Effect of fourteen daily doses of HCB on male rat body weight. N=6. Vertical bars indicate SEM. 65

15 Effect of fourteen daily doses of HCB on volume of ejaculate in the rat. N=6. Vertical bars indicate SEM. 66

16 Effect of fourteen daily doses of HCB on sperm count in the rat. N=6. Vertical bars indicate SEM 67

17 Effect of fourteen daily doses of HCB on percent morphologically abnormal sperm in the rat. N=6. Vertical bars indicate SEM. 68

18 Effects of different routes of administration of MMS upon mouse testicular DNA synthesis 92

19 Effects of MMS upon mouse testicular DNA synthesis 96

20 Effects of DNR upon mouse testicular DNA synthesis 97

21 Effects of CHL upon mouse testicular DNA synthesis 101

22 Effects of TCE 2 upon mouse testicular DNA synthesis 104

23 Effects of TCE 1,2 upon mouse testicular DNA synthesis 108

24 Effects of TCE 1,1 upon mouse testicular DNA synthesis 111

25 Effects of DCE 1,2 upon mouse testicular DNA synthesis 114

26 Comparisons of the effects of CHL; DCE 1,2; TCE 1,1; TCE 1,2; and TCE 2 upon testicular DNA synthesis, viewed on a millimolar dose basis. 115

27 Comparisons of the effects of CHL; DCE 1,2; TCE 1,1; TCE 1,2; and TCE 2 upon testicular DNA synthesis, viewed on a log(millimolar dose) basis 116

TABLES

Number — Page

1 Dosing Levels for the Multigeneration Reproduction Study of DBCM and TCM. 18

2 Reproductive Data for Female Rats Mated to Males Treated with 2,4-Dinitrotoluene 26

3 Reproductive Data for Female Rats Mated to Males Treated with 1,2,3,4-Tetrabromobutane 34

4 Reproductive Data for Female Rats Mated to Males Treated with Kepone®. 39

5 ^{14}C-Kepone® Distribution in the Male Rat Reproductive Tract. 44

6 Effects of Intra-peritoneal Administration of Kepone® on Rat Sperm Morphology 46

7 Effect of Kepone® (1x10^{-6}M) on the Distribution and Kinetics of Radiocalcium in the P388D$_1$ cells. 55

8 Reproductive Data for Female Rats Mated to Males Treated with Hexachlorobenzene. 59

9 Average Terminal Body Weight of Mice Ingesting DBCM and TCM. 69

10 Survival of Mice Ingesting DBCM and TCM. 70

11 Severity of Gross Liver Pathology of Mice Ingesting DBCM and TCM. 71

12 Reproductive Indices by Litter of Mice Ingesting DBCM and TCM. 72

13 Effects of Parental Ingestion of DBCM and TCM on the Average Number of Pups per Litter at Birth 74

14 Effects of Parental Ingestion of DBCM and TCM on Litter Survival Indices 75

15 Postnatal Body Weight Among Offspring of Mice Ingesting DBCM 76

16 Postnatal Body Weight Among Offspring of Mice Ingesting TCM 78

17 Results of Dominant Lethal Screening in Mice Ingesting DBCM 79

18 Results of Dominant Lethal Screening in Mice Ingesting TCM 80

19 Results of Teratology Screening in Mice Ingesting DBCM 81

20 Results of Teratology Screening in Mice Ingesting TCM 82

21 Incidence of Fetal Developmental Variations in the F/1c Litters of Mice Ingesting DBCM and TCM 83

22 Incidence of Fetal Developmental Variations in the F/2b Litters of Mice Ingesting DBCM and TCM 84

23 Summary of Effects of DBCM and TCM 86

24 Effects of Different Routes of Administration of MMS upon Mouse Testicular DNA Synthesis 90

25 Statistical Analysis of the Effects of Different Routes of Administration of MMS upon Mouse Testicular DNA Synthesis 91

26 Effects of MMS upon Mouse Testicular DNA Synthesis 93

27 Statistical Analysis of Effects of MMS upon Mouse Testicular DNA Synthesis 94

28 Effects of CHL upon Mouse Testicular DNA Synthesis 99

29 Statistical Analysis of Effects of CHL upon Mouse Testicular DNA Synthesis 100

30 Effects of TCE 2 upon Mouse Testicular DNA Synthesis 102

31 Statistical Analysis of Effects of TCE 2 upon Testicular DNA Synthesis 103

32 Effects of TCE 1,2 upon Mouse Testicular DNA Synthesis 106

33 Statistical Analysis of Effects of TCE 1,2 upon Mouse Testicular DNA Synthesis. 107

34 Effects of TCE 1,1 upon Mouse Testicular DNA Synthesis . . . 109

35 Statistical Analysis of Effects of TCE 1,1 upon Mouse Testicular DNA Synthesis. 110

36 Effect of DCE 1,2 upon Mouse Testicular DNA Synthesis. . . . 112

37 Statistical Analysis of Effects of DCE 1,2 upon Mouse Testicular DNA Synthesis. 113

38 mg/kg Doses and Equivalent Millimolar/kg Doses for the Halocarbons Studied 117

39 Compound Comparisons - Inhibition of Mouse Testicular DNA Synthesis. 119

LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

A, ANOVA	-- Analysis of variance (ANOVA). The variance between dose groups is compared with the variance within dose groups. p values $\leqq 0.05$ indicate a significant difference between dose groups. Dunnett's and Duncan's tests should be viewed only if the ANOVA yields a $p \leqq 0.05$.
ara-c	-- cytosine arabinoside
CHL	-- trichloroacetaldehyde (chloral hydrate)
Ci	-- curies; that quantity of radioactive nuclide disintegrating at the rate of 3.700×10^{10} atoms per second
CLI	-- Corpora Lutea Index = $\frac{\text{total No. of corpora lutea}}{\text{total No. of pregnant females}}$
cpm	-- counts per minute; the external indication of a scintillation spectrometer which is designed to enumerate ionizing events.
d	-- day(s)
DBCM	-- dibromochloromethane
DCE 1,2	-- 1,2-dichloroethane
DDW	-- distilled deionized water
$DF\geqq1$	-- $\frac{\text{total number of females with one or more dead fetuses}}{\text{total number of females with zero dead fetuses}}$
$DF\geqq2$	-- $\frac{\text{total number of females with 2 or more dead fetuses}}{\text{total number of females with less than 2 dead fetuses}}$
DF/LF	-- $\frac{\text{total number of dead fetuses}}{\text{total number of live fetuses}}$
DL	-- dominant lethal

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DLI	-- Dominant Lethal Index $= \frac{(1 - \text{number of live implants per test female})}{\text{number of live implants per vehicle control female}} \times 100$
Dn	-- Duncan's multiple range test. This test groups the dose groups according to their mean values and orders them, i.e. if two dose groups are labelled A, then they do not differ significantly from one another. It one dose group is labelled A and another is labelled B, then the two groups do differ significantly from one another, and the mean of dose group A is greater than the mean of dose group B.
DNA	-- deoxyribonucleic acid
DNAMICG	-- total micrograms of DNA in the final supernatant fraction which is a measure of old plus newly synthesized DNA
DNP	-- 2,4-dinitrophenol
DNR	-- daunorubicin
DNT	-- 2,4-dinitrophenol
dpm	-- disintegrations per minute; calculated from cpm with the use of quench correction curve
DPMH, DPMSl, DPMSF	-- the average dpm of the homogenate, first supernatant and final supernatant fractions, respectively.
Dt	-- Dunnett's t-test. Mean of the control group is compared to the mean of the non-zero dose groups for the given fraction. Significant deviations from the control (zero dose group) are indicated by a plus (+), $p \leq 0.05$
FI	-- Fetal Index $= \frac{\text{total No. of living fetuses}}{\text{total No. of pregnant females}}$
FyI	-- Fertility Index $= \frac{\text{total number of females pregnant}}{\text{total number of females inseminated}} \times 100$
g	-- grams
GI	-- Gestation Index $= \frac{\text{number of females with live pups}}{\text{number of females pregnant}} \times 100$

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g/m^3	-- grams per cubic meter
HCB	-- hexachlorobenzene
hr	-- hour(s)
IC$_{50}$	-- that concentration which causes a 50% inhibtion of the given effect.
II	-- Implantation Index $= \frac{\text{total No. of implantation sites}}{\text{total No. of pregnant females}}$
i.p.	-- intraperitoneal administration
i.t.	-- intratesticular administration
Kepone®	-- decachloro-octahydro-1,3,4-metheno-2H-cyclobuta-(cd)pentalene-2-one; chlordecone
kg	-- kilogram(s)
l	-- liter(s)
LD$_{50}$	-- that dose which causes lethality in approximately 50% of the animals to whom it is administered
LDNAMICG	-- log$_{10}$(DNAMICG)
LDPMH	-- log$_{10}$(dpm of the homogenate fraction) which is a measure of total testicular counts and equals tritiated thymidine pool size plus tritiated thymidine incorporated into newly synthesized DNA (PCA-soluble + PCA-precipitable counts)
LDPMS1	-- log$_{10}$(dpm of the first supernatant fraction) which is a measure of ^{3}H-TdR pool size (PCA-soluble counts)
LDPMSF	-- log$_{10}$(dpm of the final supernatant fraction) which is a measure of newly synthesized DNA (PCA-precipitable counts)
LI	-- Lactation Index $= \frac{\sum_{i=1}^{N} \frac{(\text{day 21 litter size})_i}{(\text{day 4 litter size})_i}}{N}$, N = number of litters
LR	-- linear regression of the dose-response curve of the given fraction. Values under LR in Tables 25, 27, 29, 31, 33, 35, and 37 are p values for obtaining a zero slope of the dose-response curve. $p \leq 0.05$ is significant.

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LSC -- liquid scintillation counting

M -- molarity = moles/liter

mg -- milligram(s)

mg/ml -- milligrams per milliter

MI -- Mating Index

$$= \frac{\text{total number of females pregnant}}{\text{total number of females mated}} \times 100$$

min -- minute(s)

ml -- milliliter(s)

MMS -- methyl-methanesulfonate

MTD -- Maximum Tolerated Dose; that concentration which causes a deviation in mean animal weight gain greater than ± 10% of the vehicle control values

N -- number of observations

nm -- 10^{-9} meters; nanometer(s)

OD -- optical density; a measure of turbidity of a solution as determined by use of a spectrophotometer

o.g. -- oral gavage route of administration

p -- probability

PBS -- phosphate buffered saline solution

PCA -- perchloric acid

PILI -- Pre-implantation Loss Index

$$= \frac{\text{total No. corpora lutea - total No. implantation sites}}{\text{total number of pregnant females}}$$

p.o. -- *per os*; drinking water route of administration

ppm -- parts per million

RI -- Resorption Index

$$= \frac{\text{total No. of fetal deaths}}{\text{total No. of pregnant females}}$$

SD -- standard deviation; is a measure of the spread of the individual measurements of a normally distributed variable

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SEM	-- standard error of the mean; equals the standard deviation of the population divided by the square root of the population size
TBB	-- 1,2,3,4-tetrabromobutane
TCE 1,1	-- 1,1,1-trichloroethane
TCE 1,2	-- 1,1,2-trichloroethane
TCE 2	-- 1,1,2-trichloroethylene
TCM	-- trichloromethane (chloroform)
TEM	-- triethylenemelamine
μCi	-- 10^{-6} Curie(s)
UDS	-- unscheduled DNA synthesis
μg	-- microgram(s); 10^{-6}grams
μg/l	-- micrograms per liter
VI	-- Viability Index
wk	-- week(s)
w/v	-- weight per unit volume

Viability Index:

$$= \sum_{i=1}^{N} \frac{\frac{(\text{day 4 litter size})_i}{(\text{day 0 litter size})_i}}{N} \quad , N = \text{number of litters}$$

SYMBOLS

C	-- degrees centigrade
^{14}C	-- an unstable isotope of carbon containing 8 neutrons in its nucleus
^{45}Ca	-- an unstable isotope of calcium containing 25 neutrons in its nucleus
^{51}Cr	-- an unstable isotope of chromium containing 27 neutrons in its nucleus
F/0	-- parental stock of mice used in a multigeneration reproduction study

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F/1a -- progeny of the first mating of the F/0 parentals

F/1b -- progeny of the second mating of the F/0 parentals

F/1c -- progeny of the third mating of the F/0 parentals

F/2a -- progeny of the first mating of the F/1b offspring of the F/0 parentals

F/2b -- progeny of the second mating of the F/1b offspring of the F/0 parentals

^{3}H-TdR -- tritiated thymidine

KCN -- potassium cyanide

NaCl -- sodium chloride

pH -- negative $\log_{10}$ of the hydronium ion concentration of a given solution; a neutral solution has a pH of 7.0; an increase in acidity causes a decrease in pH while an increase in alkalinity causes an increase in pH

x^2 -- Chi-Square

$\% \pm SEM^N$ -- average percent of control response ± standard error of the mean; control response is defined as 100%. N indicates the number of animals used to obtain the mean.

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A special note of appreciation to Mrs. Ellen Cardlin and Mr. Barry Riddle who expended considerable effort in compiling and presenting the results of the studies described in this report.

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SECTION 1

INTRODUCTION

The following compounds were chosen for study because of (1) their presence in some of the municipal water supplies tested by the EPA, (2) their concern to the EPA, (3) the paucity of data concerning their potential to induce adverse reproductive effects, and (4) their current commercial uses.

2,4-Dinitrotoluene (DNT) is an industrial intermediate used in dye manufacturing, in the production of polyurethane foams, as a gelatinizing and water proofing agent, and in the production of explosives (Short *et al.*, 1979). DNT has been found in the effluent of munitions plants (Parrish, 1977), and is an environmental hazard. It is rapidly absorbed through the intact skin, the lungs, and the gastrointestinal mucosa of man (Olishifski and McElroy, 1971). It is capable of producing a methemoglobinemia in rats, mice, and dogs (Short *et al.*, 1979) and man (Olishifski and McElroy, 1971) which can lead to headache, weakness, nausea, dizziness, drowsiness, shortness of breath, and collapse. This condition can remain for 24 to 72 hours post-exposure. When administered sub-chronically to rats, mice, and dogs, DNT produces testicular atrophy, aspermatogenesis, decreased muscular coordination, decreased appetite, weight loss, gliosis and demyelination in the brain, and hematopoietic disturbances (Ellis *et al.*, 1978; Chemical Industry Institute of Toxicology, 1978; Ellis *et al.*, 1976). Chronic exposures in rats (24 months, ≤ 32 mg/kg/day) caused the development of hepatocellular carcinomas (Ellis *et al.*, 1978). The National Cancer Institute has reported that DNT (10 mg/kg/day, 78 weeks) is capable of causing benign skin and mammary tumors in male and female rats, respectively (Department of Health, Education and Welfare, 1978). Other reported effects of DNT are the induction of cystic renal tumors (Short *et al.*, 1979), a significant increase in the frequency of chromatid gaps in lymphocytes of rat kidney cultures, and a significant increase in the percentage of dead implants in rats (≅ 90-100 mg/kg/day, 13 weeks) (Hodgson *et al.*, 1976).

Taken from: Lane *et al.*, in press.

1,2,3,4-Tetrabromobutane (TBB) is the bromination product of butadiene and is potentially useful in the synthesis of brominated polymers.

Kepone® (decachlorooctahydro-1,3,4-metheno-2H cyclobuta (cd) pentalen-2-one, chlordecone) is a highly stable polycyclic, chlorinated compound (Figure 1) which has been successfully used as an insecticide (Eversole, 1971). It has been identified as the causative agent responsible for neuropathologic symptoms, liver damage, and sterility among male workers

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involved in its production (Cohn et al., 1978). Sterility was generally observed in workers exhibiting blood Kepone® levels greater than 1 ug/ml. Testicular biopses of these patients revealed a generalized maturational arrest of the sperm even though the testerone levels were normal (Anderson, et al., 1976). The number of motile sperm increased as the blood Kepone® level decreased. Kepone® adversely affects reproduction in quail (Eroschenko and Wilson, 1975) and mice (Huber, 1965; Good et al., 1965), decreasesd egg production in laying hens (Naber and Ware, 1965), and induced generalized fetotoxicity in rodents (Chernoff and Rogers, 1976). Previous studies in this laboratory (unpublished) have demonstrated the presence of Kepone® in rat ejaculate up to 72 hours following a single oral dose, however, its effects on or association with sperm cells are not known.

Taken From: Simon et al., in press.

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The production of hexachlorobenzene (HCB) increased markedly in recent years, from 0.8 metric tons in 1966 to 6.9 metric tons in 1971 (National Academy of Sciences, 1975). HCB is widely used as a seed dressing in wheat for prevention of fungal diseases and is a reported contaminant in organochlorine pesticides (Gilbertson and Reynolds, 1972). HCB has been found in samples of ocean and river water (Seltzer, 1975; Yamato et al., 1975). Gilbertson and Reynolds (1972) have reported the presence of HCB in the eggs of common terns, presumably due to their consumption of contaminated fish. Brady and Siyali (1972) found HCB residues in human adipose tissue and milk. Experimentally administered HCB is stored in the adipose tissue of rats, dogs, and cows and persists for several months (Borzelleca et al., 1971). Over 3000 cases of human cutaneous porphyria in Turkey during 1955-1959 have been attributed to the accidental ingestion of HCB-contaminated grain (Cam and Nigogosyan, 1963). HCB has also been shown to cause neuropathy (DeMatteis et al., 1961), liver damage (Medline et al., 1973), and cancer in Syrian golden hamsters (Cabral et al., 1977). Grant et al., (1977) reported that the continual administration of HCB in the feed of male and female weanling rats at levels up to 640 ppm (≅ 60 mg/kg/day) decreases lactation, fetal body weights, and fetal viability.

Taken From: Simon et al., 1979.

Dibromochloromethane and trichloromethane (chloroform) have been identified as major contaminants in many finished municipal drinking water supplies, with levels reaching as high as 116 µg/l and 311 µg/l (USEPA, 1976).

Chloral (trichloroacetaldehyde) is used in the spraying and pouring of polyurethanes and has been found in six out of ten municipal water supplies tested by the EPA (USEPA, 1975a). The LD_{50}'s of chloral in mice, rats, and dogs have been established, but a lack of data concerning chronic exposures at low levels precludes any estimate of effects under these conditions.

1,2-Dichloroethane has several commercial uses: in the manufacture of vinyl chloride and tetraethyl lead, as an insecticidal fumigant, in

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PAGE NUMBER
EPA Form 2350-4 (4-80)
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tobacco flavoring, as a constituent of paint, varnish, and finish removers, as a metal degreaser, in soap, scouring compounds, wetting and penetrating agents, and in chemical syntheses and ore flotation (USEPA, 1975c). Twenty-six out of eighty water supplies tested by the EPA contained 1,2-dichloroethane at concentrations less than 0.2-6 ug/liter (USEPA, 1975a). 1,2-dichloroethane has been reported as a weak mutagen in S. typhimurium and DNA polymerase-deficient E. coli.

1,1,2-Trichloroethane is used as a solvent for fats, oils, waxes, and resins and also in the synthesis of organic chemicals (USEPA, 1975c). It is a by-product of water chlorination (USEPA, 1975c). Of ten water supplies tested by the EPA, only one was found to contain TCE 1,2. In addition, the finished water of New Orleans was found to contain less than 0.1-8.5 ug/liter (USEPA, 1975b). 1,1,2-trichloroethane has been reported as having the potential to be as carcinogenic as carbon tetrachloride (Browning, 1965) which has been found to induce hepatomas in mice, hamsters, and rats (Confer and Stenger, 1965; Kawasaki, 1965; Della-Porta, et al., 1961).

Trichloroethylene is used primarily in metal degreasing. It is also used as a dry-cleaning solvent, in organic syntheses, and in refrigerants and fumigants and is formed in small quantities as a by-product of water chlorination (USEPA, 1975c). Five of the ten cities studies by the EPA contained trichloroethylene at concentrations of 0.1-0.5 ug/liter (USEPA, 1975a). Effects in man appear to be CNS depression, incoordination, and unconsciousness. The compound has not been found to be very toxic to humans at the level of 100 ppm/8-hour work day.

SECTION 2

CONCLUSIONS

2,4-DINITROTOLUENE (DNT)

No dominant lethal effects were caused by the oral administration of either 60 or 180 mg DNT/kg/day for five days in rats. The 60 mg/kg/day dose produced no adverse changes in male reproductive performance. The moderately adverse effects of 180 mg/kg/day on mating and fertility indices were reversible. Severe reproductive and moderate dominant lethal effects were seen at the 240 mg/kg/day level. These effects persisted for at least eight weeks, indicating that this dose is severely debilitating.

Taken From: Lane *et al*., in press.

1,2,3,4-TETRABROMOBUTANE (TBB)

The oral administration of TBB at 10 and 40 mg/kg/day for five days in rats failed to produce the classical picture of dominant lethal effects elicited by the positive control, triethylenemelamine (TEM). TEM caused a mutagenic response in the first few weeks of mating. TBB induced significant effects in weeks 6 and 7, but these were reversed by week 14 of mating and were not considered significant evidence of dominant lethality. TBB, at both doses, impaired male reproductive performance (mating index), but not as severely as the positive control, TEM.

Taken From: Simon *et al*., 1978.

KEPONE®

Evaluation of Kepone® in a dominant lethal study (oral administration for five days in rats at 3.6 and 11.4 mg/kg/day) demonstrated no significant changes in either male fertility or dominant lethal mutations. The doses administered produced tremors as previously reported in the literature.

Following a single oral administration (40 mg/kg, 2.5 μCi/ml), Kepone® and/or its metabolites were found to distribute in the ejaculate via the seminal vesicle and appear to bind to spermatozoa.

Taken From: Simon *et al*., 1978.

Both i.p. (18 and 36 mg/kg, single acute dose) and p.o. (9 mg/kg/day for 14 days) delivery of Kepone® produced an increase in the percent of morphologically abnormal sperm. The effect of orally administered Kepone® on sperm morphology persisted for up to 7 weeks post dosing.

Kepone® interfered with cellular energy production in P388D$_1$ cells in a manner similar to the classical uncoupler of oxidative phosphorylation, dinitrophenol (DNP). Kepone® was approximately 80 times more potent than DNP in inhibiting cellular proliferation and stimulating oxygen consumption, suggesting that the mitochondria may be the ultimate site of subcellular toxicity. Kepone® also significantly altered the mitochondrial calcium distribution and the phagocytic process in the P388D$_1$ cell.

Taken From: Carmines _et_ _al_., 1979.

HEXACHLOROBENZENE (HCB)

HCB at 70 and 221 mg/kg/day p.o. for five days in rats did not induce any classical dominant lethal events during weeks 1-5 of mating as do classical mutagenic agents such as TEM. Statistically significant effects during weeks 10-14 of mating were inconsistent in their extent and direction and were attributed to weekly fluctuations in the test groups. Male reproductive performance (mating index) was impaired by HCB in a dose-related manner.

Taken From: Simon _et_ _al_., 1979.

Fourteen daily oral administrations of 35 mg HCB/kg did not significantly alter male body weight, ejaculate volume, sperm count, and the percent of morphologically abnormal sperm for up to 7 weeks post dosing.

DIBROMOCHLOROMETHANE (DBCM) AND TRICHLOROMETHANE (TCM)

In a two generation study, impairment of reproduction was observed among mice ingesting 4 mg/ml DBCM or 5 mg/ml TCM (nominal doses of 685 and 855 mg/kg/day, respectively). Significant decreases were observed in fertility, litter size, and adult female survival during gestation for both compounds at these levels. Inconsistent decreases were observed in postnatal survival and body weight. No significant adverse effects were observed for either halomethane in dominant lethal and teratology screening studies.

Taken From: Carmines _et_ _al_., 1979.

INHIBITION OF MOUSE TESTICULAR DNA SYNTHESIS

Chloral hydrate (CHL)

Doses of CHL from 10 mg/kg to 900 mg/kg were administered intratesticularly. The 600 and 900 mg/kg doses caused a significant increase in total testicular counts remaining at the time of sacrifice, one-half hour after the intratesticular injection of tritiated thymidine. These data imply a mechanism of transport of tritiated thymidine out of the testes. This mechanism is inhibited at these doses of CHL, causing a greater amount of total testicular counts, relative to vehicle control values.

Doses greater than or equal to 75 mg/kg caused a significant inhibition of testicular DNA synthesis. The amount of testicular DNA synthesis varied from 30% to 3% of control values at 75 and 300 mg/kg, respectively.

1,1,2-Trichloroethylene (TCE 2)

Doses of TCE 2 from 24 to 1000 mg/kg were tested for their effects. Intratesticular injections greater than or equal to 500 mg/kg caused an increase in tritiated thymidine pool size accompanied by a decrease in testicular DNA synthesis. The amount of testicular DNA synthesis at 500, 700, and 1000 mg/kg was 40%, 18%, and 2% of the vehicle control values, respectively.

1,1,2-Trichloroethane (TCE 1,2)

Concentrations of TCE 1,2 ranging from 3.8 to 1200 mg/kg were administered intratesticularly, and tested for their effects upon mouse testicular DNA synthesis. A dose-related increase in tritiated thymidine pool size and decrease in testicular DNA synthesis was observed. These deviations from control values became statistically significant at doses greater than or equal to 300 mg/kg. At 1000 mg/kg, DNA synthesis was inhibited to 2% of the vehicle control value.

1,1,1-Trichloroethane (TCE 1,1)

Two doses of TCE 1,1 were tested, 100 and 1000 mg/kg. Both doses caused an increase in tritiated thymidine pool size and a decrease in testicular DNA synthesis. The amounts of DNA synthesis at these doses were 60% and 27% of vehicle control values, respectively.

1,2-Dichloroethane (DCE 1,2)

DCE 1,2 was administered over a dose range of 4.9-250 mg/kg. The only significant effect observed was elicited at 250 mg/kg where a decrease in DNA synthesis to 47% of vehicle control values was effected.

Compound Comparisons (CHL; DCE 1,2; TCE 1,1; TCE 1,2; TCE 2)

In order to contrast the relative capabilities of the above compounds to inhibit testicular DNA synthesis, their dose-response curves were compared. The mean percent DNA synthesis versus log(millimolar dose/kg) was plotted. Four of the five compounds, CHL; DCE 1,2; TCE 1,2;and TCE 2 were found to have approximately equal slopes, although the curve for CHL was to the left of the others. These observations suggest that these four compounds may be causing their effects through the same mechanism which is most sensitive to CHL. CHL differs from the other compounds in that it contains an aldehyde group. This could explain the marked inhibition of testicular DNA synthesis. As only two doses of TCE 1,1 were tested, conclusions should not be drawn about the shape of the TCE 1,1 dose-response curve.

SECTION 3

RECOMMENDATIONS

A review of the research activities supported by EPA Grant No. R804290 prompts the following recommendations.

1. The effects on reproductive performance and development are highly specific and could be influenced by effects of the test chemicals on other systems (e.g. nervous system, endocrine, etc.). A holistic approach to the evaluation of potential toxicity should be undertaken. Animals receiving the same dose of the test material could be evaluated more extensively and simultaneously. For example, using the same experimental conditions, the CNS and immunological effects could be determined with reproductive (including genetic) effects. This approach is more realistic since effects produced by any chemical (drug) rarely affect one system in isolation.

2. Dominant lethal studies should be conducted at levels comparable to 10 time the level of possible human exposure to the given compounds.

When computing the corpora lutea index (= total number of corpora lutea/total number of females pregnant) in dominant lethal studies, the data from individual females should be kept separate so that inter-animal variances may be defined. The parameter as stated gives no indication of the number of corpora lutea per individual female. As a result, animals with numbers of corpora lutea very different from the mean have an exaggerated effect upon the paramter.

The preimplantation, fetal, resorption, and implantation indices were also calculated in this manner which ignores inter-animal variances.

Although 90% pure Kepone® was used to stimulate human exposure, it is suggested that the Kepone® be purified and the impurities be isolated. The pure fraction should then be used in studies which can identify the compounds eliciting the effects now labelled as being caused by Kepone® in these studies.

3. In addition to the whole animal studies, attention should be directed to molecular mechanisms of action. A thorough understanding at this level could facilitate further evaluation, aid in improving predictiveness of data and minimize unnecessary experimentation. For example, we have shown that 240 mg 2,4-dinitrotoluene/kg administered orally for five days to rats causes adverse effects on male reproduction. The nature of this effect should be determined. Several experimental approaches

could be employed to answer this question. Since it is currently believed that gene mutation is a result of DNA adduct formation with either the parent compound or metabolites of the parent compound, a DNA-adduct test may be the most direct method of quantitating potential DNA damage. Other experimental methods such as sister chromatid exchange could also be used to measure other types of DNA damage. The results of these tests would then be considered with the results observed in the whole animal reproduction studies.

The results of the multigeneration studies reported in this paper also support the need for further analyses of DNA damage and its subsequent repair. These results indicate that none of the compounds tested caused significant dominant lethal effects. But this can be a result of either damage followed by repair of the damage or properties related to the distribution and/or transformation of the agent. Only an examination of the amounts of DNA damage and repair will permit differentiation between these widely different primary effects of compound administration which yield the same secondary effect - no induction of dominant lethal mutations.

In addition to offering a molecular approach to the study of DNA damage, sister chromatid exchange formation, unscheduled DNA synthesis, adduct formation, and DNA single strand break analysis also offer the advantages of taking less time, consuming fewer man hours, and being less costly than the multigeneration studies. For these reasons, is is strongly recommended that the results presented in this report be further correlated with future studies in these suggested areas.

4. If it is found that DNA damage has been induced at those concentrations which cause no dominant lethal effects, then a repair of the damaged DNA can be inferred. In order to quantitate the amount of damaged DNA remaining after a given dose of a xenobiotic, it is important to measure DNA repair as well as DNA damage. Several techniques have been developed for this determination, and two of the procedures are amenable to studies utilizing reproductive tissues. These techniques measure unscheduled DNA synthesis and disappearance of DNA single stranded breaks resulting from DNA damage. Both procedures have been successfully employed in male reproductive tissue making these tests an important extension of the multigeneration studies.

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SECTION 4

MATERIALS AND METHODS

TEST STRAINS

The following strains of test animals were used throughout these studies:

Mice - ICR Swiss Albino, obtained from Flow Laboratories, Dublin, VA.

Rats - Sprague Dawley Albino, obtained from Flow Laboratories, Dublin, VA.
- CD Albino, obtained from Charles River Laboratories, Wilmington, MA.

TEST MATERIALS

The following test materials were obtained from the sources, and in the purity noted:

Cytosine arabinoside (ara-C)
- gift of the National Cancer Institute

Daunorubicin (DNR)
- Cancer Chemotherapy National Service Center

Decachloro-octahydro-1,3,4-metheno-2H-cyclobuta-(cd)-pentalen-2-one (Kepone®; chlordecone)
- Allied Chemical Corp., Morristown, N.J.
- 90% pure

Dibromochloromethane (DBCM)
- Columbia Organic Co.
- 99% pure

1,2 - Dichloroethane (DCE 1,2)
- Aldrich Chemical Co., Milwaukee, Wisc.
- lot #120487
- m.w. 98.96
- ≧ 99% pure

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PAGE NUMBER
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2,4-Dinitrophenol (DNP)

- MCB (Matheson, Coleman, and Bell Manufacturing Chemists)
- 98% pure

2,4-Dinitrotoluene (DNT)

- Pfaltz and Bauer, Stamford, Conn.
- crystallized to a constant melting point from diethyl ether

Hexachlorobenzene (HCB)

- Pfaltz and Bauer, Stamford, Conn.
- crystallized to a constant melting point from diethyl ether

Methyl-methanesulfonate (MMS)

- Eastman Organic Chemicals Co.
- lot #A8A
- ≧ 98% pure

Potassium cyanide (KCN)

- Baker Chemical Co., Phillipsburg, N.J.
- analyzed reagent grade
- lot #22419
- 98.5% pure

1,2,3,4-Tetrabromobutane (TBB)

- Aldrich Chemical Co., Milwaukee, Wisc.
- 99% pure by gas chromatography

Trichloroacetaldehyde (Chloral hydrate, CHL)

- Baker Chemical Co., Phillipsburg, N.J.
- lot #925086
- m.w. 165.42
- melting point analysis, 97.8° ± 2°C

1,1,1 - Trichloroethane (TCE 1,1)

- Aldrich Chemical Co., Milwaukee, Wisc.
- lot #120557
- m.w. 133.41
- 95% pure

1,1,2 - Trichloroethylene (TCE 2)

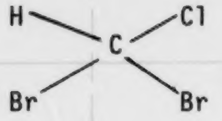
- Baker Chemical Co., Phillipsburg, N.J.
- lot #713839
- m.w. 121.39
- ≧ 99% pure

Trichloromethane (chloroform, TCM)

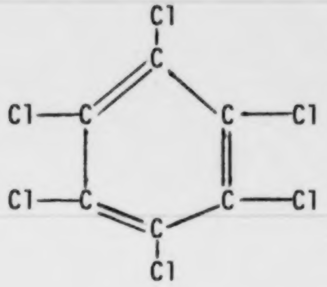
- Fischer Scientific Co.
- 99% pure

Triethylenemelamine (TEM)

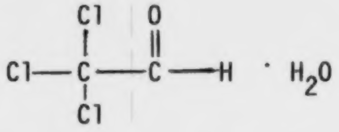
- Lederle Research Laboratories, Bethesda, MD
- 99% pure



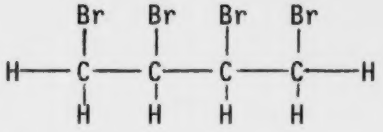
Dibromochloromethane



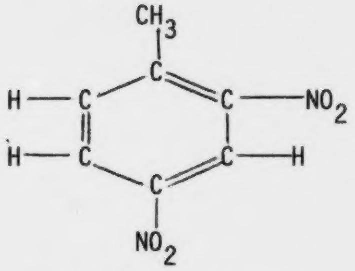
Hexachlorobenzene (HCB)



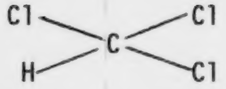
Trichloroacetic Acid
Adlehyde, hydrate
(Chloral hydrate, CHL)



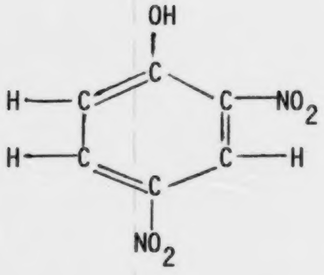
1,2,3,4-Tetrabromobutane
(TBB)



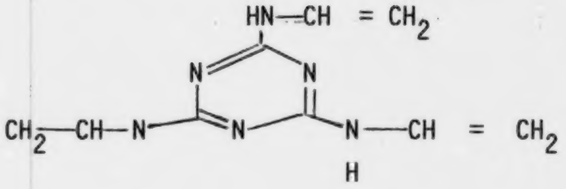
2,4-Dinitrotoluene
(DNT)



Trichloromethane
(Chloroform, TCM)

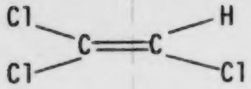


2,4-Dinitrophenol
(DNP)

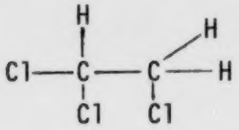


Triethylenemelamine
(TEM)

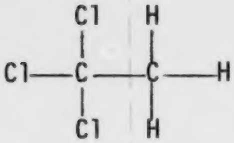
Figure 1. Structures of the compounds tested. (continued)



1,1,2-Trichloroethylene
(TCE 2)


H
H
Cl
C
C
H
Cl
Cl

1,1,2-Trichloroethane
(TCE 1,2)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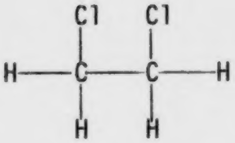
1,1,1-Trichloroethane
(TCE 1,1)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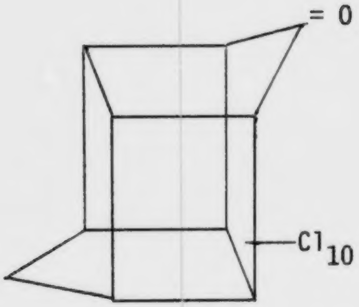
1,2-Dichloroethane
(DCE 1,2)



Decachloro-octahydro-1,3,4-metheno-2H-cyclobuta-(cd)pentalen-2-one
(Kepone®)

Figure 1

SECTION 5

EXPERIMENTAL PROCEDURES

DOMINANT LETHAL STUDIES

Adult male Sprague Dawley rats (Flow Laboratories, Dublin, VA), in groups of ten each received corn oil (Mazola®, vehicle control) or test material in corn oil by daily oral gavage for either five or ten days. Another group of ten rats received a single oral dose of triethylenemelamine (TEM, positive control, 0.5 mg/kg) in corn oil. For each test material, two or three dose levels, selected on the basis of preliminary range-finding studies, were administered. The rats were allowed Purina Rodent Chow #5001 and water *ad libitum*. Males were weighed daily during the period of compound administration and weekly thereafter.

Upon completion of the dosing, each male was allowed to mate with two naive, nulliparous females (same strain and supplier) per week for fourteen consecutive weeks. Insemination was determined by the microscopic examination of daily vaginal smears for sperm.

Females were sacrificed on day fourteen of gestation, their uteri and ovaries exposed and examined for numbers of total, living, and dead implantations and corpora lutea. A number of parameters were calculated in each dominant lethal study. These values are defined in the List of Abbreviations and Symbols as well as in the legends to the tables in which they are used.

A number of statistical tests were performed on these indices. Descriptions of the specific tests appear in the footnotes to the actual data in the Results Section. Results were considered to be significant when $p \leqq 0.05$.

COLLECTION OF EJACULATE

The ejaculate of the rat was obtained by electroejaculation (Scott and Dzuik, 1959). The unanesthetized rat was placed on a restraining board in the supine position, and a lubricated bipolar probe with electrodes 25.4 mm apart was inserted into the rectum until the longer electrode was approximately even with the fourth lumbar vertebra. A 5V, 30 Hz square wave applied to the probe generally yielded noncoagulated ejaculate while causing the animal no apparent discomfort. The volume of ejaculate obtained in this manner rarely exceeded 0.05 ml. In the event that coagu-

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lation of the ejaculate did occur, the plug was liquified with 1% α-chymotrypsin at 37 C. The collected ejaculate was used in the determination of chemical effects on sperm morphology or in studies to determine the distribution profiles of radio-labeled materials.

ANALYSIS OF EJACULATE AND EXAMINATION OF SPERM MORPHOLOGY

For the analysis of ejaculate, two groups of six adult male rats each received either test material in corn oil (Mazola®) or corn oil by daily oral gavage for 14 days. The following were determined prior to dosing and at weekly intervals for 14 weeks: body weight, volume of ejaculate, sperm count, and percentage of total sperm having abnormal morphology. Sperm were counted on a Coulter particle counter, calibrated with a hemocytometer.

Mature male CD albino rats (Charles River Laboratories, Wilmington, MA) were used for the analysis of compound effects upon sperm morphology. The animals were divided into groups of six, each group receiving either corn oil (Mazola®) or test material in corn oil, administered as a single intraperitoneal dose. The dose was repeated after resting the animals for one day. Ten days after the initial dosing, the animals were sacrificed, and their testes were removed and placed in a NaCl solution (0.9% w/v, 37 C). The epididymidmes were isolated and cut into 2-3 mm thick sections. The two epididymal minces from each rat were maintained in 3.0 ml of the warmed saline. 0.5 ml of 1% aqueous eosin was added to each suspension to enhance visualization of the sperm. Sperm from each rat were examined microscopically for morphological anomalies (400x, Hoffman modulation). The techniques of Wyrobek and Bruce (1975) were used for indexing variations in sperm morphology.

DISTRIBUTION STUDIES

^{14}C-labelled test materials in corn oil (Mazola®) were administered to adult male Sprague Dawley rats (Flow Laboratories, Dublin, VA) in a single oral gavage dose. Four animals were sacrificed at each time point, and samples of body fluids and tissues, particularly those of the reproductive system, were obtained, weighed, and oxidized. The oxidation products were counted on a Beckman LS-150 liquid scintillation counter. Mean mg equivalent test material per gram tissue was calculated for the tissues sampled at the following time points: 2, 4, 8, 12, 24, 48, and 60 hr or 1, 3, 7, 14, 28, and 84 days post-exposure.

CELLULAR SITES OF ACTION

Methodology

The P388D$_1$ cell line, originally isolated from a methylcholanthrene-induced lymphoid neophoid neoplasm (Dawe and Potter, 1956), was obtained from Dr. Dwight Stennet, University of Cincinnati, Cincinnati, Ohio.

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Cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2% 50X essential amino acids, 1% 100X vitamins, and 1% 200 mM glutamine. Cells were maintained in spinner culture and experiments were performed in 60 mm Falcon tissue culture dishes at 30 C in a humidified (98%) atmosphere of 5% CO_2 in air. In all experiments, the cells were plated at a density of 1×10^6 cells per plate and experiments were performed 48 hr later (approximately 2.5×10^6 cells/ plate). The medium was changed 18 hr prior to the beginning of all experiments. Kepone® and 2,4-dinitrophenol (DNP) were dissolved in absolute ethanol and distilled water, respectively, and then diluted 1:300 in the culture medium. The final concentrations ranged from 1×10^{-8} to 10^{-3} M. Cultures containing the same final concentrations of ethanol (0.33%) and distilled water were used as controls. The pH of the culture medium was maintained between 7.0 and 7.4. Each data point represents an average of three or more separate observations and each type of experiment reported represents a minimum of two separate experiments. The data were subjected to either analysis of variance and Tukey multiple range test or Student's t-test.

Growth Studies

Semiconfluent cells were treated with xenobiotic or vehicle for 48 hours. The tissue culture dishes were then washed four times with 3 ml phosphate-buffered saline (PBS, 37 C) to remove dead cells. One milliliter of DILUID counting solution was then added to the dish and the cells were physically removed using a rubber policeman. This cell suspension was diluted 1:10 with DILUIO® and the cells were counted using a Coulter electronic particle counter. Growth inhibition was determined by comparing the number of cells in the treated cultures with the number of cells in the vehicle cultures.

Phagocytic Assay

Semiconfluent cells were treated with xenobiotic or vehicle for 1, 2, 3, or 4 hours. Following this incubation, 0.01 ml of ^{51}Cr-labelled opsonized sheep red blood cells (Goodell et al., 1978) was added and the incubation continued for an additional 60 minutes. The medium was decanted from the dishes and 1 ml of distilled water was added to lyse the nonphagocytized sheep red blood cells. After 30 seconds, the distilled water was decanted and the tissue culture dish was washed four times with 3 ml PBS (37°C). The cells were then removed from the dishes with a rubber policeman and the radioactivity counted in a Beckman Model 300 gamma counter. The phagocytic activity was determined by comparing the counts per minute of the treated cultures with that of the vehicle controls.

Cellular Respiration

Three milliliters of a cell suspension (1×10^7 cells/ml) were placed in the sample chamber of YSI model 53 Oxygraph and a control rate of respiration was obtained. The test material was then added to the sample chamber and the rate of respiration was determined. The slopes of the changes in oxygen tension were calculated and the results were presented as percentage of the vehicle respiration rate. When oxygen consumption was being measured, 3 ml aliquots of a cell suspension (1×10^{7} cells/ml) were pretreated with 1×10^{-5} M Kepone® or vehicle for either 15, 30, 45, 60, or 75 minutes. The respiratory rate was determined as previously stated and the results are presented as a percentage of the vehicle control respiration rate.

Cellular Binding

Confluent cells were treated with 10^{-5}, 10^{-6}, and 10^{-7} M Kepone® spiked with 10^{-9} M ^{14}C-Kepone® (19.68 mCi/mmol) and incubated for either 15, 30, or 60 minutes. The cell medium (3 ml) was withdrawn, and 1 ml was added to ACS scintillant cocktail. The cells were washed four times with 3 ml PBS (4 C). One milliliter of distilled water was added to the tissue dishes and the cells were physically removed using a rubber policeman. This suspension was then added to the counting cocktail. All samples were mixed in a Vortex shaker and counted in a Beckman Model LS100 liquid scintillation system.

Calcium Efflux

The effect of Kepone® (1×10^{-6} M) on radiocalcium efflux from the cells was determined by the methods previously described by Borle (1975). Briefly, confluent cells were preincubated with either xenobiotic or vehicle for 1 hour. ^{45}Calcium (20 uCi) was added and the incubation continued for an additional hour. The tissue culture dish was then rinsed with unlabelled medium to clear the extracellular space and superficial binding sites of the isotope. Removal of the tracer from the tissue was then performed by replacing the medium at fixed intervals. This radioactive medium was then collected and added to 10 ml ACS scintillant cocktail and counted in a Beckman Model LS100 liquid scintillation system. Kinetic analysis was based on the calculations of Borle (1975). This method allowed direct calculation of the efflux rate constant, the size of several cellular calcium pools, and indirect calculation of the rate of calcium flux (the product of the pool size and rate constant). Calcium pools were identified as either cytoplasmic or mitochondrial (Warner and Carchman, submitted). Data were expressed as mean ± SEM and were analyzed using a student's t-test.

Taken From: Carmines *et al.*, 1979.

MODIFIED MULTIGENERATION REPRODUCTION STUDY

Animal Husbandry

Seven week old ICR Swiss mice (Flow Laboratories, Dublin, VA) were quarantined for two weeks, randomly grouped, and marked by either toe clipping or ear punching. The mice were housed in polycarbonate cages with sawdust bedding and were maintained on a 14-hour-light/10-hour dark cycle. The room temperature was maintained at 22-23 C with 40-60% relative humidity. The mice were allowed Purina® Rodent Laboratory Chow #5001 *ad libitum*. Females were housed three per cage, except during parturition and lactation and males were housed one per cage. During mating periods, each male was co-housed for seven days with three randomly selected females. For the dominant lethal and teratologic studies, females were examined each morning of the mating week for the presence of copulation plugs, the discovery of a vaginal plug marking day 0 of gestation. If a vaginal plug was not observed, gestational progress was estimated on the basis of abdominal distention.

Drinking Solutions

TCM (Fisher Scientific Co., ≧ 99% pure) and DBCM (Columbia Organic Co., ≧ 99% pure) were dissolved in a solution of emulphor (emulphor EL 620 = polyethoxylated vegetable oil; GAF Corp., Linden, NJ):distilled deionized water in a ratio of 1:1000. Fresh drinking solutions were prepared twice weekly according to the specifications listed in Table 1. The mice were allowed to consume the solutions ad libitum from 225 ml amber glass bottles with cork stoppers and stainless steel drinking tubes. The levels of TCM and DBCM were selected on the basis of 14-day survival and 90-day sub-chronic studies.

Experimental Design

Figure 2 outlines the modified multigeneration study which was designed to include an examination of dominant lethal and teratogenic effects of either TCM or DBCM. The test animals were continuously maintained on drinking water containing various concentrations of TCM or DBCM, vehicle, or deionized distilled water (Table 1). The F/0 mice were randomized into test groups consisting of 10 males and 30 females (two naive treatment groups were utilized), acclimated (two weeks), and placed on the test solutions. After 35 days on the test solutions, the F/0 mice were randomly mated to produce the F/1a litters. Two weeks after the last F/1a litters were weaned, the F/0 mice were re-randomized and re-mated to produce the F/1b litters. The F/1c generation was produced as described below. A 21-day litter survival study was performed on all matings. In addition, the final matings of both generations (F/1c and F/2b) were utilized for dominant lethal and teratologic studies. Except for the F/1b litter, pups were sacrificed and necropsied upon weaning. Adult weight gain and fluid consumption were monitored (weekly and twice weekly, respectively) throughout the study. Upon completion of all matings of a generation and weaning of the litters, the adults were sacrificed and necropsied.

Observations

Weight gain and fluid consumption were recorded weekly and semiweekly, respectively, for the F/0 and F/1b generations. The data were analyzed for homogeneity of initial body weights, trends in body weights and body weight gain, and trends in volume of fluid consumed and dose of the chemical ingested over time. Data analysis was performed utilizing the Statistical Analysis System (Anon, 1979).

Mating, gestation, gestation survival, and lactation survival indices, as described by Collins (1977), were calculated for each mating.

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TABLE 1
DOSING LEVELS FOR THE MULTIGENERATION REPRODUCTION STUDY OF DBCM AND TCM

Chemical	Dose Level	No. Males*	No. Females*	Concentration mg/ml
Chloroform† (TCM)	Naive Control	10	30	0.0 in DDW§
	Emulphor¶ Vehicle Control	10	30	0.0 in EDW#
	Low Dose	10	30	0.1 in EDW
	Mid Dose	10	30	1.0 in EDW
	High Dose	10	30	5.0 in EDW
Dibromochloromethane** (DBCM)	Naive Control	10	30	0.0 in DDW
	Low Dose	10	30	0.1 in EDW
	Mid Dose	10	30	1.0 in EDW
	High Dose	10	30	4.0 in EDW

* Number of males and females listed represents the original size of the parental stocks.

† Columbia Organic Co.

§ DDW - distilled deionized water

¶ Common vehicle control for TCM and DBCM

# EDW - 0.1 emulphor in DDW

** Fisher Scientific Co.

Taken from: Riddle *et al.*, in press.

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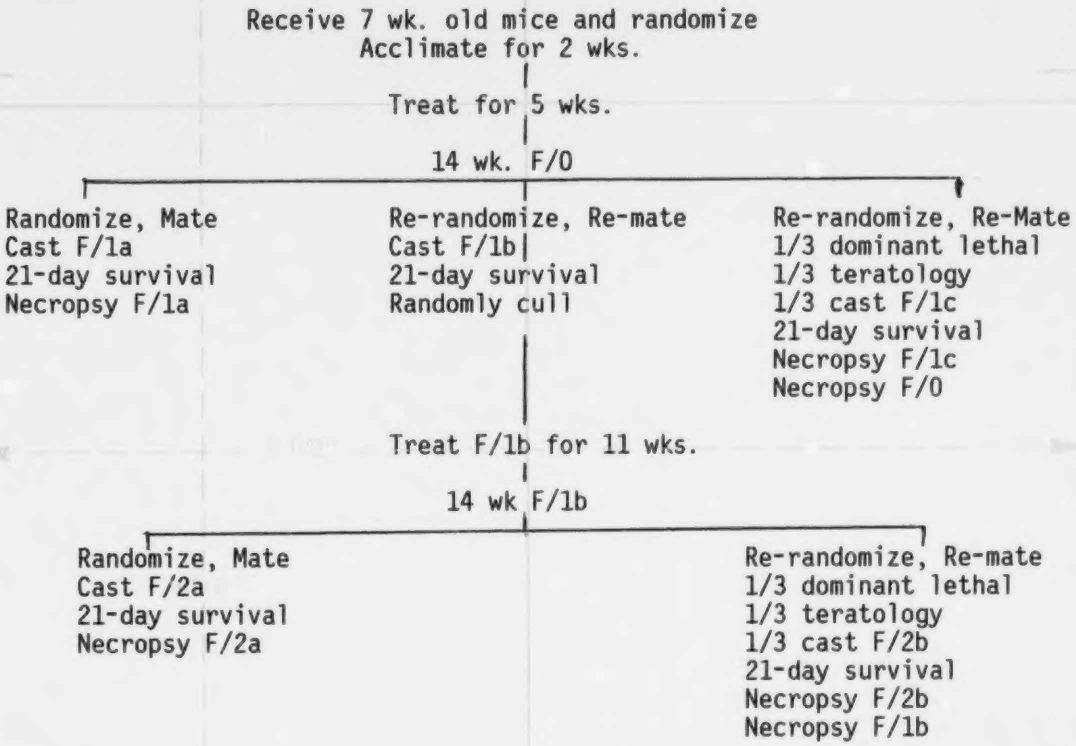
Receive 7 wk. old mice and randomize
Acclimate for 2 wks.
Treat for 5 wks.
14 wk. F/0
Randomize, Mate
Cast F/1a
21-day survival
Necropsy F/1a
Re-randomize, Re-mate
Cast F/1b
21-day survival
Randomly cull
Re-randomize, Re-Mate
1/3 dominant lethal
1/3 teratology
1/3 cast F/1c
21-day survival
Necropsy F/1c
Necropsy F/0
Treat F/1b for 11 wks.
14 wk F/1b
Randomize, Mate
Cast F/2a
21-day survival
Necropsy F/2a
Re-randomize, Re-mate
1/3 dominant lethal
1/3 teratology
1/3 cast F/2b
21-day survival
Necropsy F/2b
Necropsy F/1b

Figure 2. Flowchart for the modified-multigeneration reproduction study.

Taken from: Riddle *et al*., in press.

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The discontinuous nature of these indices required the application of nonparametric techniques for statistical analysis. The Kruskal Wallis X^2 approximation was used to test for group effects and Dunn's approximation to a distribution-free multiple comparison was used to test for localization of effects (Hollander and Wolfe, 1973).

A 21-day survival study was performed on the pups of all generations. Pups were counted on days 0, 4, 7, 14, and 21. Offspring were sexed on days 7, 14, and 21. Viability and lactation indices (Collins, 1977; Fitzhugh, 1969) were calculated. Both parametric and nonparametric procedures were utilized for the analysis of the data. The statistical sampling unit was the litter (Gaylor, 1977).

Dominant Lethal Screening

Treated males from the F/0 and F/1b generations were mated with naive females (1:3) for seven days (F/1c and F/2b matings) for the dominant lethal test (Green et al., 1977). The females were sacrificed 14 days from the midweek of co-housing, their uteri exposed, and the number of fetal implants, early and late resorptions, and viable fetuses counted. The resorption index was calculated. Non-parametric procedures were utilized for the analysis of the data. The litter was the statistical sampling unit.

Teratology Screening

Treated females from the F/0 and F/1b generations were mated (3:1) with naive males for seven days. The females were sacrificed on the eighteenth day of gestation, their uteri exposed, and the number of implants, resorptions, and live and dead fetuses counted. These findings were analyzed in a manner similar to the dominant lethal data. Fetuses were individually weighed and examined for gross defects. In the event that no gross malformations were found, every third fetus was fixed in Bouin's fluid for subsequent cross-sectioning and examination for visceral anomalies. The remainder of the fetuses were prepared for skeletal examinations by suspension of soft tissue in 1% KOH, with subsequent staining in aqueous alizarin red S (Barrow and Taylor, 1969; Wilson, 1965).

Necropsy

All mice found dead or sacrificed *in terminus* were weighed and necropsied. Litters were sacrificed at day 21 (except F/1b) with 1 male and 1 female pup from each litter randomly selected for necropsy. All tissues were carefully examined for evidence of alterations. Suspect tissues were fixed in 10% neutral buffered formalin for examination at a later date.

Taken From: Riddle *et al*., in press.

INHIBITION OF TESTICULAR DNA SYNTHESIS

For all experiments, 5-week old male ICR Swiss mice were obtained from Flow Laboratories, Dublin, Virginia, and were used at 12 weeks of age.

The test compound was administered in two equal intratesticular injections (i.t., 0.02 ml/testis, 0.04 ml/mouse). Three and one half hours later, each mouse was injected with tritiated thymidine (i.t., 10 uCi/testis, 20 uCi/ mouse). Ether narcosis was employed prior to each injection. After a pulse time of one half hour, the mice were sacrificed by cervical dislocation, the testes surgically removed, and homogenized in 1 ml PBS buffer (0.15 M phosphate buffer in 0.85% NaCl, pH 7.2). The homogenizing vessel was then rinsed with 1 ml of PBS buffer and this was added to the homogenate. In all experiments, the data obtained from individual animals were kept separate, and the animal was the smallest statistical unit of comparison.

Testicular DNA was isolated via a modification of the Shibko method (Shibko et al., 1967) (Figure 3). The procedure involves the separation of DNA, protein, and RNA through successive differential PCA precipitations and base hydrolyses. For the purposes of these experiments, aliquots of only three fractions were saved. These are the homogenate from which the amount of tritiated thymidine is calculated, the first supernatant fraction from which PCA-soluble counts, or tritiated thymidine pool size is quantitated, and the final supernatant fraction from which the PCA-precipitable counts, or newly synthesized DNA is quantitated. A DNA determination (OD 257 nm) was also made on the final supernatant fraction. Using the information obtained from a quench correction curve, cpm were converted to their corresponding dpm, and the total dpm per fraction was subsequently determined. Using an IBM System 370/158 computer, the following functions were performed on the Log_{10}(dpm) of each of the three supernatant fractions as well as the micrograms DNA in the final supernatant fraction:

plot dose-response curves
linear regression of these dose-response curves
dose-effect determination for all dose-response curves
bar chart of the means versus dose
analysis of variance
Dunnett's T-test
Duncan's multiple range test

The following functions were performed on the dpm in each of the three supernatant fractions:

determination of means
determination of standard error of the means (SEM)
expression of means ± SEM as a percent of the vehicle
    control response, defined as 100%

Because these methods quantitate both newly synthesized DNA and total testicular DNA, one could perform a statistical analysis of newly synthesized DNA per microgram of testicular DNA (dpm final supernatant/microgram DNA final supernatant = specific activity). In these experiments, however, neither the numerator nor the denominator of this fraction is a controlled variable. As a result a change in specific activity would not necessarily be solely due to the amount of newly synthesized DNA. For this

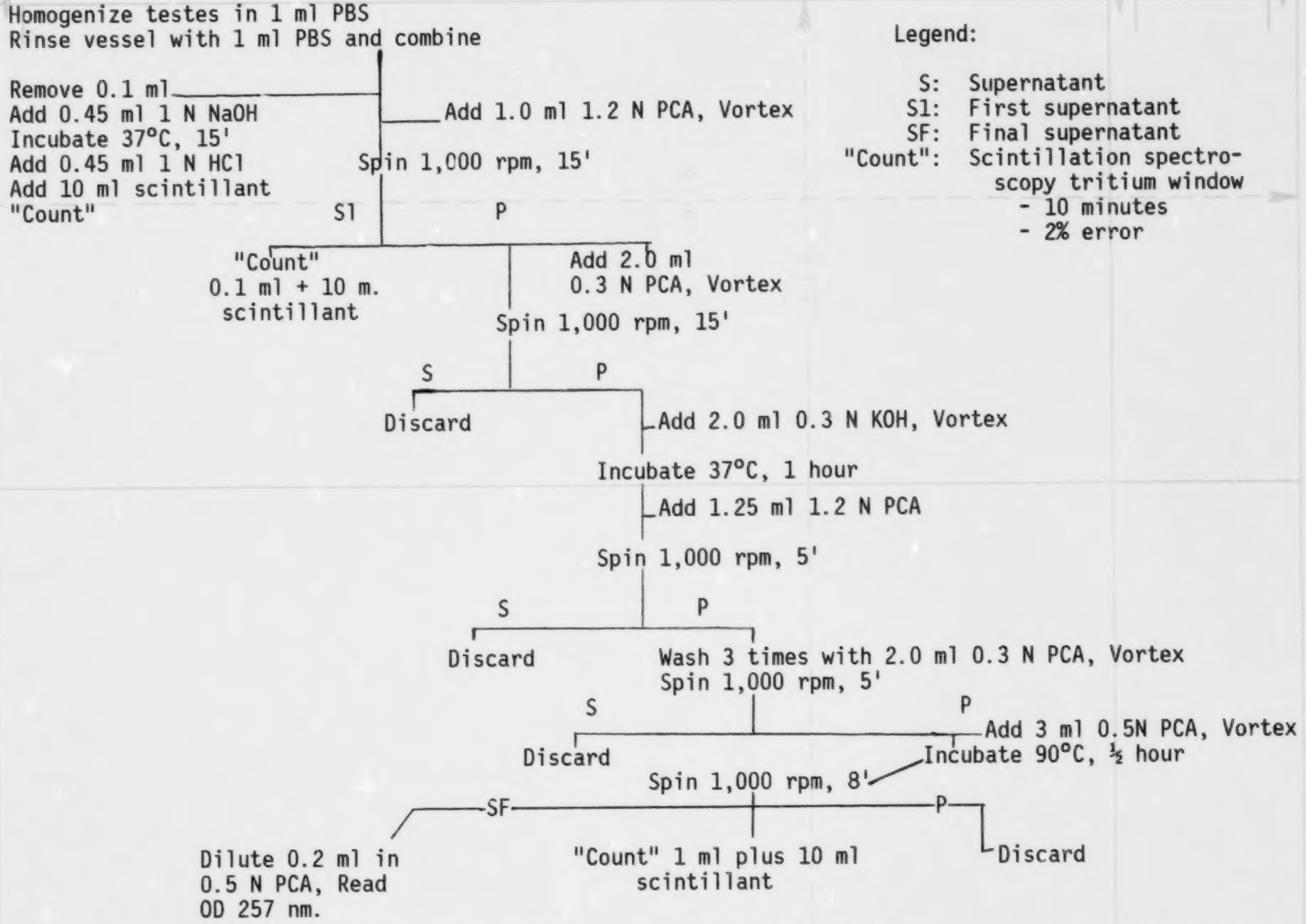
Homogenize testes in 1 ml PBS
Rinse vessel with 1 ml PBS and combine
Remove 0.1 ml
Add 0.45 ml 1 N NaOH
Incubate 37°C, 15'
Add 0.45 ml 1 N HCl
Add 10 ml scintillant
"Count"
Add 1.0 ml 1.2 N PCA, Vortex
Spin 1,000 rpm, 15'
S1
P
"Count"
0.1 ml + 10 m.
scintillant
Add 2.0 ml
0.3 N PCA, Vortex
Spin 1,000 rpm, 15'
S
P
Discard
Add 2.0 ml 0.3 N KOH, Vortex
Incubate 37°C, 1 hour
Add 1.25 ml 1.2 N PCA
Spin 1,000 rpm, 5'
S
P
Discard
Wash 3 times with 2.0 ml 0.3 N PCA, Vortex
Spin 1,000 rpm, 5'
S
P
Discard
Add 3 ml 0.5N PCA, Vortex
Incubate 90°C, ½ hour
Spin 1,000 rpm, 8'
SF
P
Dilute 0.2 ml in
0.5 N PCA, Read
OD 257 nm.
"Count" 1 ml plus 10 ml
scintillant
Discard
Legend:
S: Supernatant
S1: First supernatant
SF: Final supernatant
"Count": Scintillation spectroscopy tritium window
- 10 minutes
- 2% error

Figure 3. Isolation of mouse testicular DNA.

reason, it would be incorrect to view specific activity as a measure of testicular DNA synthesis.

The Log_{10} of the dpm in each fraction was used due to a natural skewing of data obtained from scintillation spectroscopy. Use of the Log_{10} removes some of this skewing and decreases the within-group variances resulting from this skewing. The importance of this is explained below where the analysis of variance and the other analyses performed are discussed.

The effect of dose upon the parameters measured was evaluated through analysis of the slope of the respective dose-response curves. The p value for obtaining a zero slope with the data obtained is indicated in the LR (linear regression) columns of the respective parameters analyzed (see Statistical Analysis Tables). A p value $\leqq 0.05$ indicates a significant dose effect for the parameter tested, under the conditions employed.

The analysis of variance (ANOVA) compares the ratio of the between-group variances to the within-group variances. From this ratio, a p value for randomly obtaining a ratio greater than that obtained experimentally is determined. A value $\leqq 0.05$ is significant. The sensitivity of this analysis is in part dependent upon minimizing the within-group variances. This problem has been previously addressed in the discussion of the natural skewing of LSC data and explains the importance of minimizing this variance.

In addition to the ANOVA, Dunnett's T-test and Duncan's multiple range tests were performed on the parameters studied. Because the ANOVA is least likely to indicate a false positive (indicate significance where none in fact exists), the results of these tests should be examined only if the ANOVA indicates a p value $\leqq 0.05$. If this is the case, then the results of the Dunnett's test are used to indicate significant deviation of the mean of a given dose group from that of the vehicle control. In the following tables and graphs, a significant deviation from mean control value is indicated by a plus (+).

The Duncan's multiple range test determines whether the dose groups, zero and non-zero, differ significantly from one another. The groups are ordered by their means, and different letter designations assigned to those groups which differ significantly from one another. For example, three dose groups assigned letters A, B, and C indicate that the means of all three dose groups differ significantly from one another, with the means decreasing in value from group A through group C.

SECTION 6

RESULTS AND DISCUSSION

2,4-DINITROTOLUENE (DNT)

Dominant Lethal Study

Approximately fifty-three percent of the second group of rats receiving 240 mg DNT/kg/day died within two weeks of receiving the first dose. Although the cause of each death was not rigorously determined, gross examination of the animals indicated that no deaths were due to gavaging error or disease. During the dosing period and the subsequent week, the high dose animals were cyanotic and lost weight. This was the only group which lost weight in this study (See Figure 4). The large weight gain seen between weeks 1 and 2 may have been due to a return of appetite after the initial effects of DNT had subsided. No other deaths occurred in the study, but severe cyanosis was evident in the group receiving 180 mg DNT/kg/day for five days.

The reproductive and DL effects of DNT and TEM are summarized in Table 2. 60 mg DNT administered for five days caused no significant changes in any of the indices examined. Three statistically significant effects were seen in the group receiving 180 mg DNT/kg/day for five days: increase in PILI, week 2; decrease in MI, week 5; and decrease in CLI, week 5. A number of adverse reproductive effects were observed among the rats receiving the high dose, most noticeably the MI of weeks 1-6. The low MI made it difficult to interpret the other data for those weeks. Because of the low MI at this dose, this portion of the study was continued for an additional six weeks. By the thirteenth week, all reproductive and DL indices were indistinguishable from control values. TEM produced classical DL effects for the first four weeks of mating, similar to those effects observed by other investigators (e.g., Soares and Sheridan, 1977); there were no observed effects upon either mating or fertility.

The use of DL test can help answer two questions regarding the toxicity of DNT: is it mutagenic/carcinogenic and does it affect reproductive performance? The DL data can be used to augment other mutagenicity/ clastogenicity/carcinogenicity data. The DL test is performed totally *in vivo* which allows for the testing of all metabolites produced by the organism, not just by one particular organ (usually the liver). These metabolites may be too reactive or produced in too low a concentration to be detected and studied *in vitro*. Furthermore, this test was done in a species (rat) which is sensitive to the carcinogenic effects of DNT (Chemical Industry Institute of Toxicology, 1978; Ellis *et al*., 1978). Mutagenic metabo-

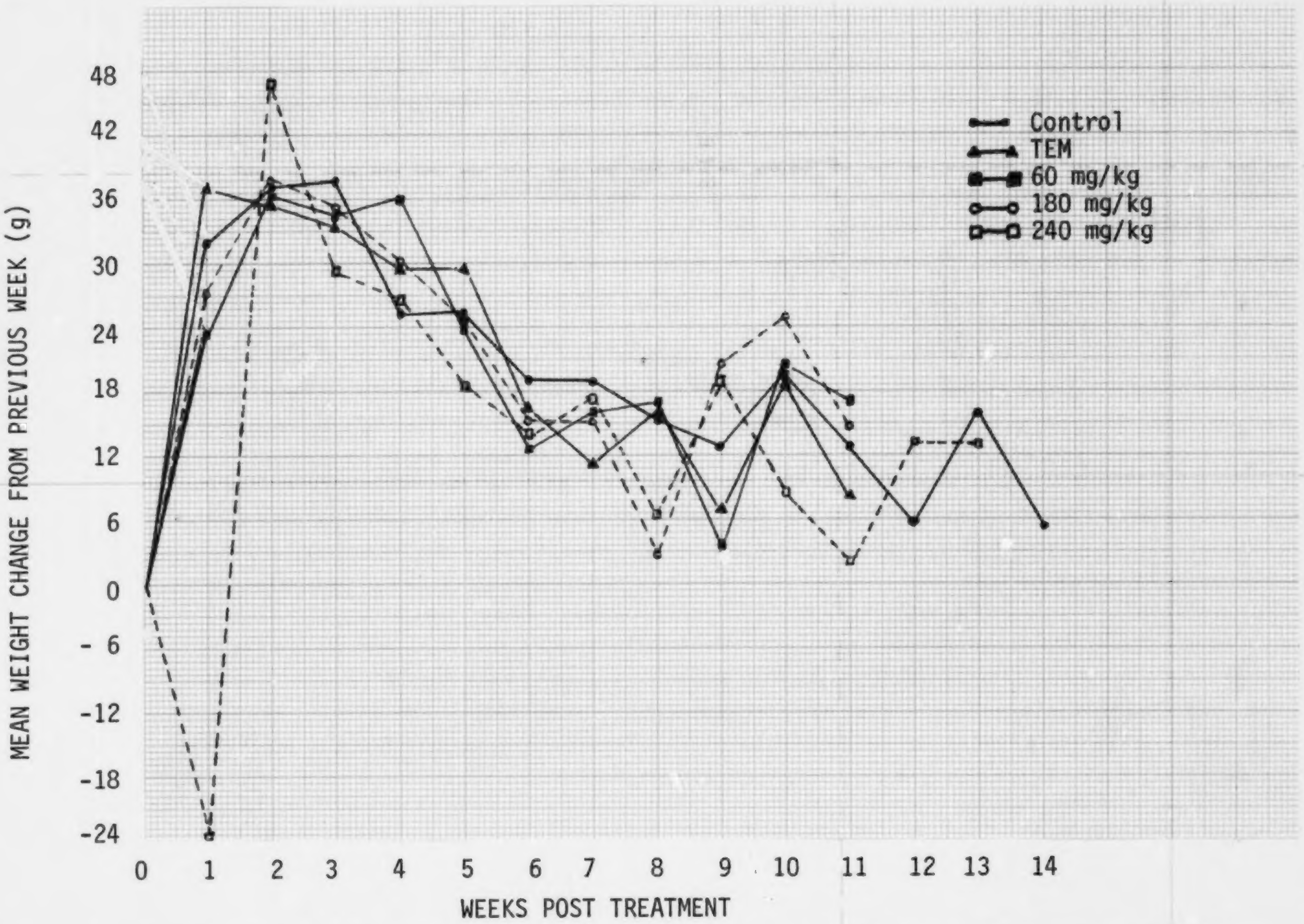

Figure 4. Mean body weight changes of male rats after receiving five oral doses of DNT in a dominant lethal study.
Taken from: Lane *et al*., in press.

TABLE 2. REPRODUCTIVE DATA FOR FEMALE RATS MATED TO MALES TREATED WITH 2,4-DINITROTOLUENE

CORN OIL CONTROL*

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	6	30	66	11.67	9.83	1.83	9.83	0.00	0/59	0/6	0/6
2	14	70	88	11.93	9.86	2.07	8.93	0.93	13/125	7/7	3/11
3	10	50	83	15.70	12.90	2.80	12.40	0.50	5/124	5/5	0/10
4	14	70	88	12.64	11.14	1.50	10.43	0.71	10/146	9/5	1/13
5	18	90	100	13.28	10.94	2.33	9.78	1.17	21/176	9/9	2/16
6	15	75	100	13.00	11.60	1.40	10.93	0.67	10/164	7/8	2/13
7	18	90	100	12.00	10.94	1.06	10.44	0.50	9/188	6/12	3/15
8	13	65	93	11.69	11.15	0.54	10.69	0.46	6/139	4/9	1/12
9	15	75	100	11.27	10.07	1.20	9.73	0.33	5/146	4/11	1/14
10	16	80	100	11.88	11.25	0.63	10.50	0.75	12/168	10/6	2/14
11	16	80	94	11.75	11.00	0.75	10.31	0.69	11/165	7/9	3/13
12	13	65	87	11.69	9.62	2.08	8.31	1.31	17/108	8/5	4/9
13	14	70	100	11.93	11.07	0.86	10.29	0.79	11/144	7/7	4/10
14	18	90	100	11.28	9.50	1.78	8.89	0.61	11/160	7/11	1/17

* Compound administered for 5 days via daily oral gavage.

(continued)

TABLE 2. (Continued)

TEM (0.5 mg/kg)†

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2	DLI
1	8	40	80	11.50	8.13	3.38	4.25	3.88¶	31/34§	8/0§	6/2§	56.77
2	11	55	92	14.09	10.00	4.09	4.82	5.18¶	57/53§	10/1	9/2§	46.02
3	12	60	80	13.67	8.75	4.92	2.83¶	5.92¶	71/34§	12/0§	9/3§	77.18
4	16	80	100	12.81	10.56	2.25	8.44	2.13	34/135§	10/6	7/9	19.08
5	16	80	100	12.31	11.25	1.06	10.56	0.69	11/169	8/8	3/13	-7.98
6	16	80	94	12.81	11.69	1.13	11.25	0.44	7/180	6/10	1/15	-2.93
7	14	70	100	12.54	11.23	1.31	10.38	0.85	11/135	8/6	1/13	0.57

† Compound administered as a single dose via oral gavage.

§ Significantly different from control (two by two contingency tables with correction for continuity, $p \leqq 0.05$.

¶ Significantly different from control (Kruskal Wallis χ^2 with Dunn's Approximation to a Distribution-free Multiple Comparison, $p \leqq 0.05$.

(continued)

TABLE 2. (Continued)

DNT (60 mg/kg)*

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2	DLI
1	8	40	100	14.75	12.25	2.50	12.00	0.25	2/96	2/6	0/8	-22.08
2	14	70	100	13.57	11.86	1.71	11.21	0.64	9/157	7/7	2/12	-25.53
3	12	60	86	14.92	10.67	4.25	10.00	0.67	8/120	6/6	1/11	19.35
4	15	75	100	12.47	10.60	1.87	10.20	0.40	6/153	4/11	1/14	2.21
5	18	90	100	12.44	11.44	1.00	10.28	1.17	21/185	9/9	1/17	-5.11
6	14	70	93	13.14	10.57	2.57	10.07	0.50	7/141	6/8	1/13	7.87
7	15	75	100	12.07	10.53	1.53	10.07	0.47	7/151	4/11	3/12	3.54

* Compound administered for 5 days via daily oral gavage.

(continued)

TABLE 2. (Continued)

DNT (180 mg/kg)

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2	DLI
1	6	30	75	12.33	9.00	3.33	8.17	0.83	5/49	3/3	1/5	16.89
2	9	45	75	15.11	9.22	6.00¶	7.89	1.33	12/71	5/4	3/6	11.65
3	8	40	80	13.13	11.50	1.63	11.00	0.50	4/88	4/4	0/8	11.29
4	9	45	75	13.11	11.33	1.78	10.89	0.44	4/98	4/5	0/9	-4.41
5	11	55¶	85	9.82¶	8.45	1.36	7.73	0.73	9/85	5/6	1/10	20.96
6	15	75	83	11.93	11.13	0.80	10.73	0.40	6/161	4/11	1/14	1.83
7	14	70	93	12.36	10.93	1.43	10.14	0.79	11/142	7/7	3/11	2.87

¶ Significantly different from control (Kruskal Wallis χ^2 with Dunn's Approximation to a Distribution-free Multiple Comparison, $p \leqq 0.05$.

(continued)

TABLE 2. (Continued)
DNT (240 mg/kg)*

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2	DLI
1	4	20¶	100	11.00	6.00	5.00	4.75	1.25	5/19	3/1	1/3	46.81
2	3	21	60	12.33	5.67	6.67	5.00	0.67	2/15	2/1	0/3	59.68
3	1	7¶	50	8.00	3.00	5.00	0.00	3.00	3/0§	1/0	1/0	100.00
4	2	14¶	100	7.00	1.50	5.50	0.00	1.50	3/0§	2/0	1/1	100.00
5	1	7¶	100	7.00	1.00	6.00	0.00	1.00	1/0	1/0	0/1	100.00
6	2	14¶	68	8.00	3.00	5.00	2.00	1.00	2/4§	2/0	0/2	80.84
7	5	36	71	11.60	5.00¶	6.60¶	5.00¶	0.00	0/25	0/5	0/5	53.23
8	6	43	68	13.50¶	10.50	3.00¶	10.00	0.50	3/60	3/3	0/6	-2.77
9	6	43	86	13.00	11.17	1.83¶	10.17	1.00	6/61	3/3	2/4	3.14
10	8	57	100	11.88	10.25	1.63	9.38	0.88	7/75	6/2	1/7	9.02
11	9	64	90	9.78	7.11	2.67	6.67	0.44	4/60	4/5	0/9	19.74
12	9	64	82	10.56	9.44	1.11	9.22	0.22	2/83	2/7	0/9	10.40
13	10	71	91	11.80	10.90	0.90	10.50	0.40	4/105	3/7	1/9	-18.11

* Compound administered for 5 days via daily oral gavage.

§ Significantly different from control (two by two contingency tables with correction for continuity, $p \leq 0.05$.

¶ Significantly different from control (Kruskal Wallis χ^2 with Dunn's Approximation to a Distribution-free Multiple Comparison, $p \leq 0.05$).

Taken from: Lane *et al.*, in press.

lites may be produced in the rat and not seen in mutagenicity tests using other species. Effects on reproductive function can be simultaneously determined with little additional effort.

Despite the advantages of the DL test, there are several limitations. Potential DL effects might not be seen (false negative results) if the reactive metabolite(s) produced by the organism is (are): too reactive to reach the germinal cells; excluded from the germinal cells by the blood-testis barrier; incapable of causing chromosomal damage or rearrangement, which are the effects measured by the DL test (Green *et al.*, 1976; Epstein *et al.*, 1972). Additionally, the carcinogenicity of DNT may involve epigenetic mechanisms, which would limit the predictiveness of the DL (or any other mutagenicity) test. Finally, any damage to sperm DNA may be of such a type that it is repaired by the ova (Generoso *et al.*, 1979).

False positive DL mutations could be observed which are indirect consequences of DNT exposure. The anoxia caused by the methemoglobinemia could conceivably cause damage to germinal cells which could lead to DL mutations. Secondly, the apparent anorexia which was caused by the DNT exposure could have damaged germinal cells in varying stages of maturation which could lead to DL effects weeks after the nutritional deficit had occurred. Other experiments would have to be done to resolve these questions.

However, the DL test may be an appropriate method for testing the mutagenicity/clastogenicity of DNT. There is some evidence to support the carcinogenicity of DNT, but little evidence for the effects of DNT on point mutations (i.e., multiple negative responses in the *Ames/Salmonella* assay and in the rat primary hepatocyte culture UDS assay, Couch *et al.*, 1979; Chiu *et al.*, 1978; Cotruvo *et al.*, 1977; Simmon *et al.*, 1977). Therefore, either chromosomal or epigenetic mechanisms would appear to be involved in the carcinogenic response to DNT. As discussed in the introduction, the chromosomal data has been mostly negative, but not enough work has been done in this area to produce a definite answer. An attempt to resolve the genetic/epigenetic mechanism of carcinogenicity has only recently been described (Reitz *et al.*, 1980), and has not been applied specifically to DNT at this time. Epigenetic mechanisms are usually associated with chemicals which produce severe toxicity and cell death without binding to DNA in the organs in which the tumors arise. This possibility has not been fully excluded in the case of DNT, but firm denial or confirmation of this point must await further work. Finally, unpublished data from our laboratory shows that DNT and/or metabolites enter the reproductive tract of male rats, supporting the findings of Ellis *et al.*, (1976). Therefore, the DL assay appears to be suitable for studying the mutagencity/clastogenicity of DNT.

Our results indicate that 60 mg DNT/kg/day for five days produced no adverse reproductive or DL effects. No signs of toxicity were seen other than slight cyanosis.

The next highest dose used, 180 mg DNT/kg/day for five days, produced cyanosis, but only three reproductive or DL values which were statistically different from control. The MI (week 5) which was statistically significant from control occurred at the same time that the control MI reached its highest value. Also, the MI values for this dose of DNT increased steadily throughout the seven weeks of mating, so that the one week was not specifically affected. It is concluded that the MI is only moderately affected by DNT at a dose level of 180 mg/kg/day for five days and that the effect is temporary. The significant CLI (week 5) change cannot be attributed to the effect of DNT and is considered to be a statistical fluctuation. The increase PILI (week 2) could be due to DNT, but appears to be again due to statistical fluctuation. No overall pattern was observed which could be construed as DNT causing dominant lethality at this dose level. These findings suggest that cyanosis causes no DL effect.

Weight loss, cyanosis, and death occurred during the week after dosing male rats with 240 mg DNT/kg/day for five days. The number of deaths which occurred was much greater than was expected from the range finding study. The reason for this is not known.

Adverse reproductive and DL effects were also seen at the high dose of DNT. All indices that were tabulated were affected, MI and DF/LF most severely. Although the FyI was reduced for most of the study, no statistically significant values were obtained. This finding constrasts with that of Soares and Lock (1979), who reported a significant reduction in male mouse fertility at a comparable dose (250 mg/kg/day for two days). Most of the changes observed during the first six weeks are probably due to the effects of DNT on male reproductive function and are not specifically DL effects. The low number of pregnant females made statistical analysis of the results difficult to interpret. However, there does appear to be a suggestion of DL effects in the early weeks of the study. Evidence for this conclusion can be seen especially in the DF/LF and DL indices. However, other important indices were also altered (e.g., II, PILI, FI).

After week 7 of the study (i.e., the extended portion), the MI had increased (i.e., was not significantly different from control) to make the other data more statistically meaningful. II, PILI, and FI were significantly altered in week 8. Thereafter, only the FILI of weeks 9 and 10 were statistically different from control values. It should be noted that these indices were different from control values before week 8, but were not statistically significant. These indices are the most relevent to DL testing (Green _et al_., 1976). However, the spectrum of effects was not similar to that produced by the positive control (TEM). By week 11, there appeared to be little difference between treated and control data. It may be concluded that 240 mg DNT/kg/day for five days has slight DL and major reproductive effects in rats and that these effects are slowly reversible.

DNT is reported to cause aspermatogenesis (Ellis _et al_., 1976). This makes it difficult to deduce exactly which cells of the spermatogenic cycle are primarily affected by the chemical. The problem is compounded by the fact that fertility is not severely affected by DNT at any time. In order to cause pregnancy, mature spermatozoa must be present in the vasa deferentia and epididymis. Therefore, some spermatogenesis must be occurring in the testes of the treated rats, but the rate is not known. One cannot determine which type(s) of cell(s) was (were) affected during dosing by extrapolating back from the time DL effects were seen.

An interesting characteristic of the data presented is the sharp increase in DL between 180 and 240 mg DNT mg/kg/day. Two explanations are possible. The low number of pregnant females in the high dose group could bias the results in favor of a positive DL response. Although this explanation cannot be discounted, it is not necessarily the only answer. The possibility that dosimetry is an important factor in determining DL mutations has been raised by Sega *et al.*, (1974). These authors showed that the DNA of mouse spermatozoa dosed with ethyl methanesulfonate was alkylated at lower doses than when DL effects were observed. This indicates that a certain minimum amount of damage to the DNA is required before any DL effects are seen. The implication is that a threshold exists for DL effects below which no effects will be seen. Our data indicates that this threshold is between 180 and 240 mg DNT/kg/day for five days.

The results presented support published carcinogenicity data (except the NCI bioassay) but almost none of the mutagenicity data (see Introduction) concerning purified DNT. In particular, our DL results concur with the rat data reported by Hodgson *et al.*, (1976), but not the mouse data reported by Soares and Lock (1978, 1979). The species difference could be due to the differences in absorption of DNT from the gastrointestinal tract (Hwang *et al.*, 1976) since metabolism appears to be the same in both species (Short *et al.*, 1979; Hwang *et al.*, 1976). Although our results indicate a weak genetic toxic effect of DNT, the high dose used and the general toxicity associated with this dose indicate that the issue of the mutagenicity/clastogenicity/carcinogenicity of DNT has not been resolved.

Further work may help to clarify some of the problems involved with DNT exposure. More data are needed to characterize the reproductive effects of high levels of DNT. Histopathological examination of the testes may elucidate the nature of the effects of DNT on spermatogenesis. Weekly sperm counts could also be useful. *In vitro* cell transformation tests and binding to DNA may help to clarify the mutagenic/clastogenic/carcinogenic effects of DNT.

Taken From: Lane *et al.*, in press.

1,2,3,4-TETRABROMOBUTANE (TBB)

Dominant Lethal Study

The maximally tolerated dose of TBB in rats was determined to be 40 mg/kg/day for 10 days. There was no significant effect of TBB upon weight gain. In a preliminary study, the administration of 80 mg/kg per day of TBB for 10 days produced a significant decrease in weight gain that persisted for several weeks.

Table 3 summarizes the effects of treatment with TBB upon nine reproductive parameters during the 14 sequential matings. The high dose of TBB

TABLE 3
REPRODUCTIVE DATA FOR FEMALE RATS MATED TO MALES TREATED WITH 1,2,3,4-TETRABROMOBUTANE

CORN OIL CONTROL*

Week	Number Pregnant	MI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	12	86	13.8	11.8	2.30	10.6	0.75	9/133	3/9	2/10
2	17	94	15.3	12.6	2.90	11.8	0.88	15/199	7/10	4/13
3	12	92	13.6	12.1	1.60	11.2	0.67	8/137	4/7	2/9
4	16	89	12.5	11.0	1.37	11.0	0.12	2/174	2/14	0/16
5	14	82	12.6	11.4	1.23	11.0	0.43	6/154	4/10	1/13
6	13	100	13.4	12.7	0.69	11.9	0.77	10/155	6/7	3/10
7	11	79	12.5	11.9	0.64	11.5	0.36	4/127	3/8	1/10
8	7	58	12.4	10.4	2.22	9.7	0.71	5/68	2/5	2/5
9	11	94	13.1	11.3	1.81	10.9	0.36	4/120	3/8	2/9
10	16	94	14.0	12.6	1.62	12.1	0.44	7/194	4/12	3/13
11	15	94	13.7	11.6	2.13	10.9	0.73	11/163	7/8	3/12
12	17	94	12.1	10.5	1.53	9.8	0.82	14/166	6/11	3/14
13	16	89	13.7	10.9	2.68	10.5	0.37	7/168	5/11	1/15
14	14	93	13.3	10.9	2.46	10.2	0.61	8/143	5/8	1/12

* Compound administered for ten days via daily oral gavage.

(Continued)

TABLE 3 (Continued)

TEM 0.5 mg/kg†
Positive Control

Week	Number Pregnant	MI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	6	46§	11.2¶	6.6¶	4.70	4.0¶	2.67¶	16/24§	5/1	5/1§
2	6	46§	12.3	5.8¶	2.50	3.8¶	2.00	12/25§	4/2	3/3
3	9	90	12.0	8.5¶	3.50	3.4¶	5.10¶	46/31§	8/1§	8/1§
4	11	69	12.8	10.7	2.10	8.1¶	2.60¶	29/104§	9/2§	8/3§
5	10	71	13.0	11.0	2.00	10.8	0.20	2/108	2/9	0/11
6	12	92	11.9	11.7	0.50	11.6	0.17¶	2/138	12/0§	11/1§
7	10	90	12.9	11.1	1.70	10.8	0.30	3/108	3/7	0/10
8	12	80	13.9	12.0	2.83	11.1	0.42	5/134	3/9	1/11
9	10	100	13.2	11.8	1.50	11.5	0.30	3/115	3/6	0/9
10	13	93	13.8	10.9	2.92	10.8	0.08	1/141	1/12	0/13
11	11	92	13.4	10.5	3.00	10.0	0.54	6/110	4/7	1/10
12	11	92	13.0	10.7	2.27	10.3	0.36	4/114	4/7	0/11
13	14	100	13.2	12.7	0.57	11.8	0.71	10/166	6/8	4/11
14	12	80	12.7	10.7	2.00	10.4	0.33	4/125	4/8	0/12

† Compound administered as a single dose via oral gavage.

§ Significantly different from control (χ^2 with Yates' correction, $p \leq 0.05$)

¶ Significantly different from control (Student's T-test, $p \leq 0.05$)

(Continued)

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TABLE 3 (Continued)

TBB 10 mg/kg/day*

Week	Number Pregnant	MI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	10	67	14.2	12.9	1.50	12.5	0.40	4/125	4/6	0/10
2	16	94	14.4	13.3	1.87	11.6	0.94	15/181	5/11	3/13
3	16	100	13.9	12.2	1.62	11.9	0.37	6/177	4/12	1/15
4	15	83	13.9	11.5	2.20	11.0	0.47	7/165	3/12	2/13
5	11	86	12.8	12.3	0.45	12.1	0.18	2/133	2/9	0/11
6	12	86	12.1	10.7¶	1.42	10.6	0.17	2/126	2/10	0/12
7	10	83	11.4	8.6¶	2.80¶	8.5¶	0.10	1/85	1/9	0/10
8	12	100	13.7	11.4	2.33	10.6	0.75	9/128	5/7	3/9
9	13	81	16.6	12.5	4.58¶	11.9	0.46	6/156	5/7	1/11
10	15	88	14.4	11.5	2.93	10.8	0.80	12/161	7/8	3/12
11	16	94	13.9	10.9	3.06	10.4	0.44	7/167	5/11	2/14
12	17	100	13.1	12.8	0.94	11.4	1.06	18/194	8/9	7/10
13	17	94	14.8	12.1	2.70	11.4	0.64	11/195	7/10	2/15
14	15	100	13.1	12.0	1.20	11.3	0.42	11/169	5/9	1/13

* Compound administered for ten days via daily oral gavage.

¶ Significantly different from control (Student's T-test, p ≦ 0.05)

(Continued)

TABLE 3 (Continued)

TBB 40 mg/kg/day*

Week	Number Pregnant	MI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	13	76	13.8	11.6	2.41	11.5	0.54	7/144	6/75	1/12
2	14	93	14.3	12.1	2.00	11.8	0.21	3/167	3/11	0/14
3	16	100	13.8	12.1	1.75	11.6	0.44	7/186	5/11	2/14
4	13	81	12.5	10.8	1.69	10.2	0.31	4/137	1/12	1/12
5	15	100	12.9	12.1	0.87	12.0	0.20	3/179	1/14	0/15
6	11	75	12.9	12.3	0.66	11.8	0.27	3/132	4/7	2/9
7	13	87	11.3	10.1¶	1.38	9.5¶	0.31	4/130	7/6	0/13
8	12	86	13.7	11.4	4.75¶	7.6	1.75	21/92§	6/6	4/8
9	9	90	14.7	12.3	2.33	10.8	1.56¶	14/97¶	7/2	2/7
10	16	94	16.9	12.7	4.26¶	12.1	0.75	12/192	5/11	2/14
11	19	95	14.2	11.0	3.31	10.7	0.31	6/203	5/14	1/18
12	17	89	13.7	11.9	2.00	11.3	0.53	9/193	5/11	2/14
13	17	100	13.2	12.1	1.29	10.0	2.00	34/171§	13/4§	7/10
14	16	89	14.1	11.1	3.06	10.4	0.62	10/167	7/9	3/13

* Compound administered for ten days via daily oral gavage.

§ Significantly different from control (χ^2 with Yates' correction, $p \leq 0.05$)

¶ Significantly different from control (Student's T-test, $p \leq 0.05$)

Taken from: Simon _et al_., 1978.

(40 mg/kg/day) elicited the following statistically significant effects: decreases in the numbers of implantation sites and fetal index during Week 7; and an increased proportion of nonviable to viable fetuses during Week 13. The lower dose of TBB (10 mg/kg/day) elicited the following statistically significant events: decreases in the numbers of implantation sites during Weeks 6 and 7; increased preimplantation losses during Week 7 and a decrease in the fetal index during Week 7. By Week 10, both treatment groups appeared to have recovered.

TEM, the positive control agent, elicited classical dominant lethal effects during the earliest weeks of mating (Bateman, 1966), including decreases in the proportion of pregnant females and the proportion of viable to nonviable fetuses during the first 4 weeks of the experiment. There was a significant decrease in the numbers of corpora lutea the first week and in the numbers of implantation sites during the first 3 weeks.

Most mutagens elicit their effects during the early weeks (1-5) of a dominant lethal test (Bateman, 1966). The types of effects seen with TBB reveal a potential for causing impaired reproductive performance but not dominant lethal mutations. Thus, the effects seen with TBB are qualitatively different than those caused by TEM.

Clermont and Leblond (1953) have described the time course for spermatogenesis in the rat. All of the spermatozoa involved in the significant results seen with TBB would have existed as stem-cell spermatogonia and gonocytes while TBB was being administered. The return to normal values by week 14 indicates the reversibility of the TBB effects.

Taken From: Simon *et al*., 1978.

KEPONE®

Dominant Lethal Study

Body weight gains of rats given Kepone® or triethylenemelamine (TEM) were not significantly different from those of the controls. In a preliminary study, animals receiving 18 or 36 mg Kepone®/kg daily for five consecutive days showed marked reduction in weight gain. Greater than 73% (11 of 15) mortality was seen at the higher dose.

Reproductive data for all females are presented in Table 4. TEM elicited classical dominant lethal mutations as described by Bateman and Epstein (1971). Females mated to males receiving Kepone® exhibited sporadic statistically significant differences from control in several parameters. These differences were, however, inconsistent in their occurrence as well as their extent and direction from control and were not attributed to Kepone® administration.

Kepone® did not elicit dominant lethal mutations or affect male fertility in this study. The presence of Kepone® associated with the sperm or in the ejaculate did not appear to affect ad versely any of the reproductive parameters measured. It must be emphasized that the doses of

TABLE 4. REPRODUCTIVE DATA FOR FEMALE RATS MATED TO MALES TREATED WITH KEPONE®

CORN OIL CONTROL (10 mg/kg/day)*

Week	Number Pregnant	Number Insem.	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	14	15	75	93	12.85	10.85	2.00	10.28	0.57	7/134	5/9	2/12
2	16	16	80	100	15.81	11.37	4.43	10.81	0.62	10/173	9/7	1/15
3	17	17	85	100	15.47	12.17	3.29	11.17	1.00	17/190	8/9	2/15
4	13	14	70	93	15.38	11.61	3.84	11.07	0.53	7/144	6/7	1/12
5	17	17	85	100	15.00	11.23	3.82	10.88	0.35	6/185	5/12	1/16
6	15	15	75	100	13.93	10.46	3.46	10.13	0.33	5/152	3/12	1/14
7	18	18	90	100	15.94	12.22	3.72	11.66	0.55	10/210	6/12	3/15
8	17	17	85	100	15.52	10.76	4.76	10.35	0.41	7/163	5/12	2/15
9	15	15	75	100	13.73	10.46	3.26	9.86	0.66	10/148	7/8	3/12
10	16	18	90	89	10.56	9.18	1.37	8.87	0.31	5/142	5/11	0/16
11	16		85	94	13.87	10.31	3.56	9.68	0.66	10/155	4/12	3/13
12	13	13	65	100	13.84	11.92	1.92	11.46	0.46	6/149	5/8	1/12
13	15	16	80	94	15.46	11.00	4.46	10.53	0.46	7/158	6/9	1/14
14	13	13	65	100	15.92	10.69	5.46	9.53	1.15	15/124	9/4	4/9

* Compound administered for five consecutive days via daily oral gavage.

(continued)

TABLE 4 (Continued)

TEM (0.5 mg/kg)†

Week	Number Pregnant	Number Insem.	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	10	10	50	100	13.10	11.00	2.10	6.50§	4.50§	20/86¶	10/0¶	10/0¶
2	13	14	70	93	16.84	10.69	6.15	6.92§	3.76§	49/90¶	12/1	10/3¶
3	15	16	80	94	15.80	10.20§	5.60	4.26§	5.93§	89/64¶	15/0¶	14/1¶
4	17	18	90	94	15.88	11.47	5.05	8.52	2.35	40/145¶	11/6	7/10
5	15	16	80	94	15.40	9.66	5.73	8.93	0.73	11/134	8/7	2/13
6	11	11	55	100	13.81	10.81	3.36	9.27	1.54	17/102¶	4/7	1/10
7	17	17	17	100	15.47	10.35§	5.17	9.17§	1.17	20/156¶	8/9	5/12
8	13	15	75	86	15.61	10.92	4.76	10.15	0.76	10/132	7/6	2/11
9	16	17	85	94	13.50	8.43§	5.06	7.93	0.50	8/127	7/9	1/15
10	13	16	80	81	11.23	10.15	1.69	9.46	0.69	9/123	4/9	2/11
11	16	17	85	94	16.37	11.06	5.31	10.50	0.50	8/169	7/9	1/15
12	11	14	70	79	17.27§	11.00	6.27	10.54	0.45	5/116	4/7	1/10
13	17	17	85	100	15.52	10.64	4.88	9.52	1.00	17/162	10/7	2/15
14	16	17	85	94	14.75	9.62	5.12	9.31	0.31§	5/149¶	5/11	0/16

† Administered as a single dose via oral gavage.

§ Significantly different from control (Student's T-test, $p \leqq 0.05$).

¶ Significantly different from control (χ^2 with Yates' correction, $p \leqq 0.05$).

(continued)

TABLE 4 (Continued)
KEPONE® (3.6 mg/kg/day)*

Week	Number Pregnant	Number Insem.	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	8	8	40	100	14.00	10.00	4.00	9.50	0.37	3/76	3/5	0/8
2	16	16	80	100	16.56	11.12	5.43	10.62	0.50	8/170	6/10	2/14
3	18	18	90	100	14.88	11.27	3.61	10.55	0.72	13/190	12/6	1/17
4	18	19	95	95	17.05	11.42	4.05	11.00	0.53	8/197	6/12	1/17
5	19	19	95	100	15.44	11.52	3.78	10.36	1.15	22/197¶	10/9	4/15
6	14	15	75	93	12.35	9.64	2.71	9.00	0.64	9/132	6/8	3/11
7	18	18	90	100	14.16	10.50§	3.77	9.66§	0.61	11/174	6/12	4/14
8	18	18	90	100	16.55	9.39	7.16	8.77	0.61	11/158	10/8	1/17
9	15	15	75	100	13.53	11.26	2.26	10.60	0.80	12/159	8/7	2/13
10	16	17	85	94	14.75§	10.62§	4.12§	10.12	0.50	8/162	5/11	3/13
11	18	18	90	100	15.88	11.11	4.47	10.72	0.38	7/193	5/13	1/17
12	15	16	80	94	14.26	11.06	3.20	10.26	0.80	12/159	9/6	3/12
13	16	16	80	100	15.25	10.56	4.62	10.00	0.56	9/160	6/10	2/14
14	18	18	90	100	14.83	9.61	5.22	9.27	0.33§	6/167¶	4/14¶	2/16

* Administered for five consecutive days via daily oral gavage.

§ Significantly different from control (Student's T-test, $p \leqq 0.05$).

¶ Significantly different from control (χ^2 with Yates' correction, $p \leqq 0.05$).

(continued)

TABLE 4 (Continued)
KEPONE® (11.4 mg/kg/day)*

Week	Number Pregnant	Number Insem.	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	9	10	50	90	13.44	11.22	2.44	10.11	1.11	10/91	5/4	3/6
2	13	13	65	100	14.00	11.07	3.07	10.36	0.76	10/134	7/6	1/12
3	16	16	80	100	17.37	12.18	5.37	11.68	0.50	8/187	5/11	2/14
4	16	17	85	95	17.06	11.68	5.56	10.56	1.06	17/169	9/7	6/10
5	13	14	70	93	14.84	9.92	4.92	8.76	1.15§	15/114¶	9/4	3/10
6	12	12	60	100	16.08	11.25	4.83	10.50	0.75	9/126	4/8	1/11
7	13	13	65	100	15.07	10.84§	4.23	10.15	0.69	9/132	6/7	3/10
8	15	15	75	100	14.06	11.13	2.93	10.86	0.26	4/163	4/11	0/15
9	14	15	75	93	14.42	9.85	4.57	9.42	0.42	6/132	4/10	1/13
10	13	16	80	81	13.84§	10.61§	3.30	10.07	0.53	7/131	4/9	3/10
11	15	16	80	94	14.93	11.00	3.93	10.33	0.73	10/155	5/10	4/11
12	13	13	65	100	15.61	10.61	5.76§	9.84§	0.76	10/128	6/7	3/10
13	13	15	75	87	14.38	10.53	4.61	8.61§	1.15	15/112¶	8/5	5/8
14	16	16	80	100	15.18	10.75	4.43	10.25	0.50	8/164	7/9	1/15

* Administered for five consecutive days via daily oral gavage.

§ Significantly different from control (Student's T-test, $p \leq 0.05$).

¶ Significantly different from control (χ^2 with Yates' correction, $p \leq 0.05$).

Taken from: Dougherty *et al.*, in press.

Kepone® administered in these experimental situations elicited tremors and thus was a biologically active dose.

The report of Cueto, Page and Saffiotti (1976) concerning the carcinogenic activity of Kepone® has raised concern over its mutagenic potential. However, Kepone® did not demonstrate any mutagenic activity in the present dominant lethal assay nor has it caused mutation in the Ames Assay, micronucleus assay or cytogenic test on exposed populations (Dr. M. A. Friedman, personal communication). The mutagenic effects of triethylenemelamine (TEM) used as a positive control were clearly evident (Table 4) and in agreement with results reported elsewhere (Soares and Sheridan, 1977; Green et al, 1976; and Bateman and Epstein, 1971).

The reported adverse effects of Kepone® on the male reproductive system (Larson et al., 1979; Eroschenko, 1978; Anderson et al., 1976; Eroschenko and Wilson, 1975) include oligospermia, aspermia, and loss of libido. All of these results were seen after the chronic administration of Kepone®. These effects were not observed in the acute setting. Eroschenco and Wilson (1975) have suggested that the adverse reproductive effects reported are a result of Kepone®'s estrogenic activity.

Distribution Study

All animals receiving Kepone® (40 mg/kg p.o., 2.5 μCi/ml) exhibited tremors commencing within two hours and increasing in frequency and intensity until three days following dosage. No gross pathology associated with Kepone® administration was evident at sacrifice.

The distribution of ^{14}C-Kepone® is presented in Table 5. The highest amount of radioactivity was found in the plasma. The concentration of Kepone® in the seminal vesicle reached its maximum at Day 3 as did the levels of Kepone® in the plasma, prostate, and vas deferens. Kepone® concentrations in the secretions of the seminal vesicle, washed and unwashed sperm peaked at Day 7. Plasma/tissue ratios generally declined to a minimum by Day 14 and, subsequently, increased by Day 21. However, the plasma/seminal vesicle secretion ratio still appeared to be decreasing on Day 21. Some Kepone® is associated with the spermatazoa and cannot be removed by washing with buffer.

Kepone® is well absorbed and distributed throughout the body of the rat (Egle, et al., 1978), although the partitioning of Kepone® from the plasma into various tissues differs somewhat from other organochlorine pesticides. For example, the ratio of the concentration of Kepone® in adipose tissue to plasma is 7:1 (Cohn et al., 1978) while it is 280:1 for DNT (Morgan and Roan, 1974). Thus, Kepone® remains in the blood and liver longer, and at higher concentrations, than other organochlorine pesticides. Skalsky et al. (1979) have suggested that this unusual distribution pattern may be a result of the binding of Kepone® to high density lipoproteins.

As shown in Table 5, the concentration of Kepone® remains highest in the plasma, entering the accessory glands of the male reproductive tract

TABLE 5. ^{14}C-KEPONE® DISTRIBUTION IN THE MALE RAT REPRODUCTIVE TRACT

DAYS AFTER KEPONE® ADMINISTRATION	PLASMA (ug Kepone® X 10^{-4}/ml)	SEMINAL VESICLE SECRETION (ug Kepone® x 10^{-4}/ml)	PROSTATE (ug Kepone® x 10^{-4}/mg)	VAS DEFERENS (ug Kepone® x 10^{-4}/mg)	SEMINAL VESICLE (ug Kepone® x 10^{-4}/mg)	UNWASHED SPERM (ug Kepone® x 10^{-4}/10^{6} cells)	WASHED SPERM (ug Kepone® x 10^{-4}/10^{6} cells)	% Kepone® associated with sperm
1	5229±522	39.0±9.2	2.99±0.25	2.62±0.13	1.23±0.33	1.09±0.33	0.13±0.13	11.9%
2	4897±291	80.5±16.7	3.49±0.14	2.36±0.27	1.69± .03	0.82±0.33	0.13±0.08	5.8%
3	7968±1318	82.2± 1.4	5.39±0.68	3.57±0.22	1.81±0.30	0.91±0.12	.38±0.21	41.8%
5	5628±1843	79.7±22.4	4.82±0.58	2.59±0.45	1.67±0.13	1.28±0.13	.74±0.48	57.8%
7	4208±466	99.1±18.5	3.57±0.54	2.35±0.45	1.37±0.13	2.33±0.66	.78±0.78	33.5%
14	2822±133	63.1±0.8	3.16±0.30	1.67±0.17	1.37±0.23	0.71±0.29	.31± .31	43.7%
21	2378±187	64.8±6.5	1.95±0.07	1.04±0.06	0.96±0.11	0.44±0.03	.14± .13	31.8%

Taken from: Dougherty _et al_., in press.

at much lower levels. After a single oral dose, peak concentrations of Kepone® occurred on day three in the plasma, prostate, vas deferens and seminal vesicle. The concentration of Kepone® found in the secretions of the seminal vesicle was surprisingly higher than the residue present in the tissue itself. This suggests that the secretions of the seminal vesicle offer a sink for Kepone® and would represent a major contribution of Kepone® appearing in the ejaculate.

The fluid taken from the vasa deferentia listed in Table 5 as "unwashed sperm," contains not only spermatozoa but also epididymal secretions. By passing aliquots of this material through 0.45μ filters the spermatozoa were retained on the filter, and a majority of the residual secretions were removed by further washings with buffer. A portion of the Kepone® is closely associated with the spermatozoa and is not readily washed away. The nature of this binding of Kepone® to spermatozoa remains to be elucidated.
Taken From: Dougherty _et_ _al_., 1981.

Effects on Sperm Morphology

The i.p. administration of Kepone® (18 and 36 mg/kg) to rats resulted in a dose-dependent increase in the percent of abnormal sperm (Table 6).

Fourteen daily doses (9 mg/kg, p.o.) of Kepone® to adult male Sprague-Dawley rats produced the following effects, with respect to a corn oil vehicle control group: decreases in body weight, decreases in ejaculate volume, decreases in sperm count, and increases in percent of morphological abnormalities in sperm. These effects were generally manifested 14-21 days post dosing and are summarized in Figures 5-8.

Cellular Sites of Action

Preliminary experiments suggested that Kepone® stimulated cellular respiration in a manner similar to uncouplers of oxidative phosphorylation. The effects of Kepone® and DNP (an uncoupler of oxidative phosphorylation) were compared. Figure 9 demonstrates the effect of Kepone® and DNP on the growth of the $P388D_1$ cell. Kepone® produced a dose-dependent inhibition of cell proliferation (ANOVA, p = 0.001, F = 117). The IC_{50} (inhibitory concentration 50%, Litchfield and Wilcoxon, 1949) was 3.5×10^{-6} M (1.77×10^{-5} M to 6.93×10^{-7} M, 95% confidence limits). The slope of the line was calculated as -5.4×10^{-2} (4.1×10^{-1} to 6.9×10^{-3}, 95% confidence limits). The vehicle (ethanol) had no significant effect on the growth of the cells. DNP also produced a dose-dependent (ANOVA, p = 0.001; F = 161) inhibition of cellular proliferation. The IC_{50} was 2.7×10^{-4} M (1.07×10^{-3} M to 6.78×10^{-5} M, 95% confidence limits) and the slope was -1.67×10^{-1} (5.65×10^{-1} to 4.94×10^{-2}, 95% confidence limits). The slope ratio between the two dose-response curves was 3.1 (critical value = 10.9) indicating that the two lines do not deviate significantly from parallelism. The potency ratio was 77, indicating that Kepone® was significantly more potent that DNP in inhibiting cellular proliferation.

TABLE 6
EFFECTS OF INTRA-PERITONEAL ADMINISTRATION OF KEPONE® ON RAT SPERM MORPHOLOGY*

Group	Number of Animals	Number with Abnormal Sperm	Percent Abnormal Sperm
Corn Oil Control†	6	3	0.7
Kepone®, 18 mg/kg	6	4	1.1
Kepone®, 36 mg/kg	5§	3	2.6

* During the ten days between intial dosing and sacrifice, the control group experienced a mean body weight change of 2.1% (gain), while the 18 and 36 mg/kg Kepone® groups both showed decreases in mean body weight (-1.5% and -14.9%, respectively).

† Vehicle control was Mazola® corn oil.

§ One animal from this group died after the second administration of Kepone®.

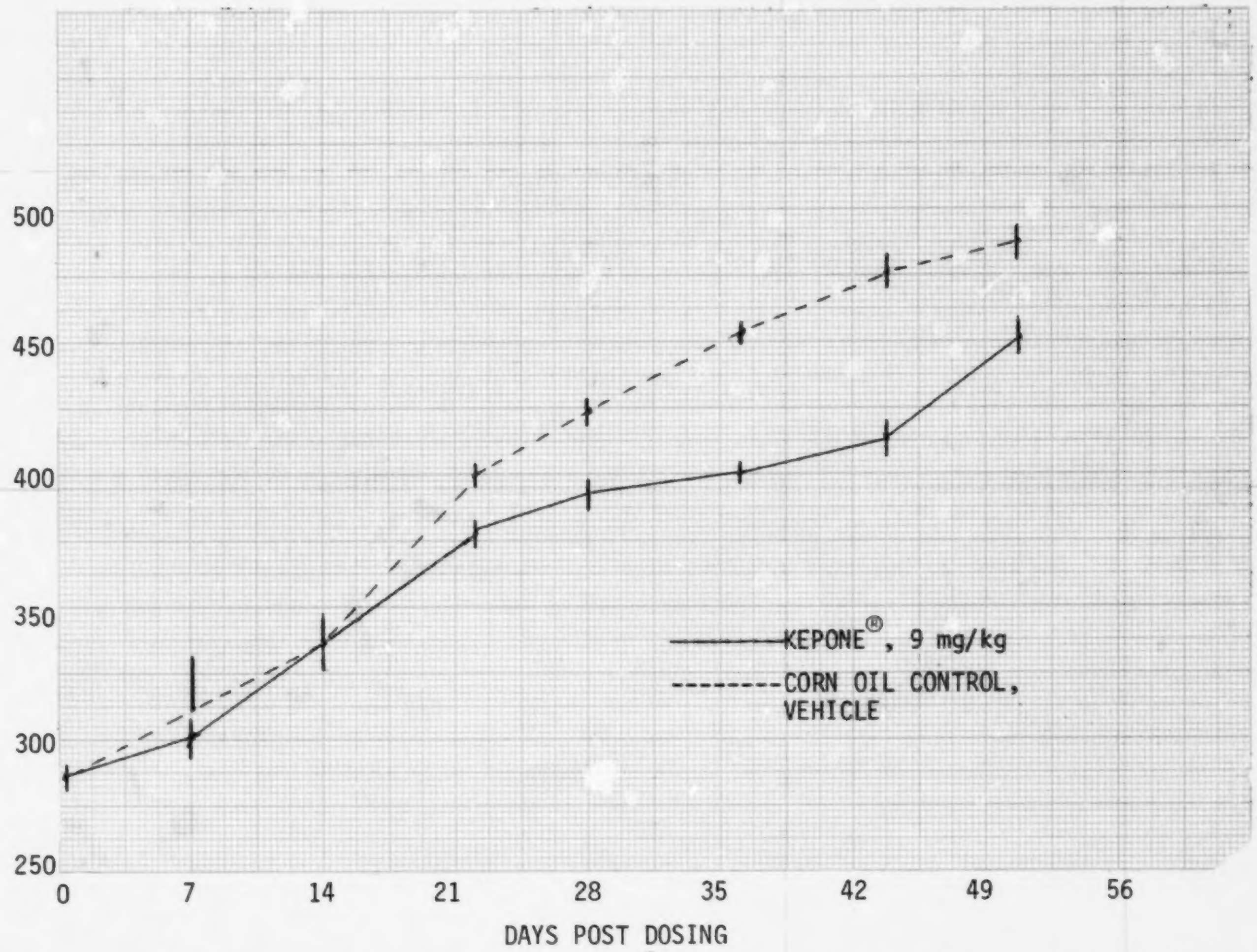

Figure 5. Effect of fourteen daily doses of Kepone® on male rat body weight. N=6. Vertical bars indicate SEM.

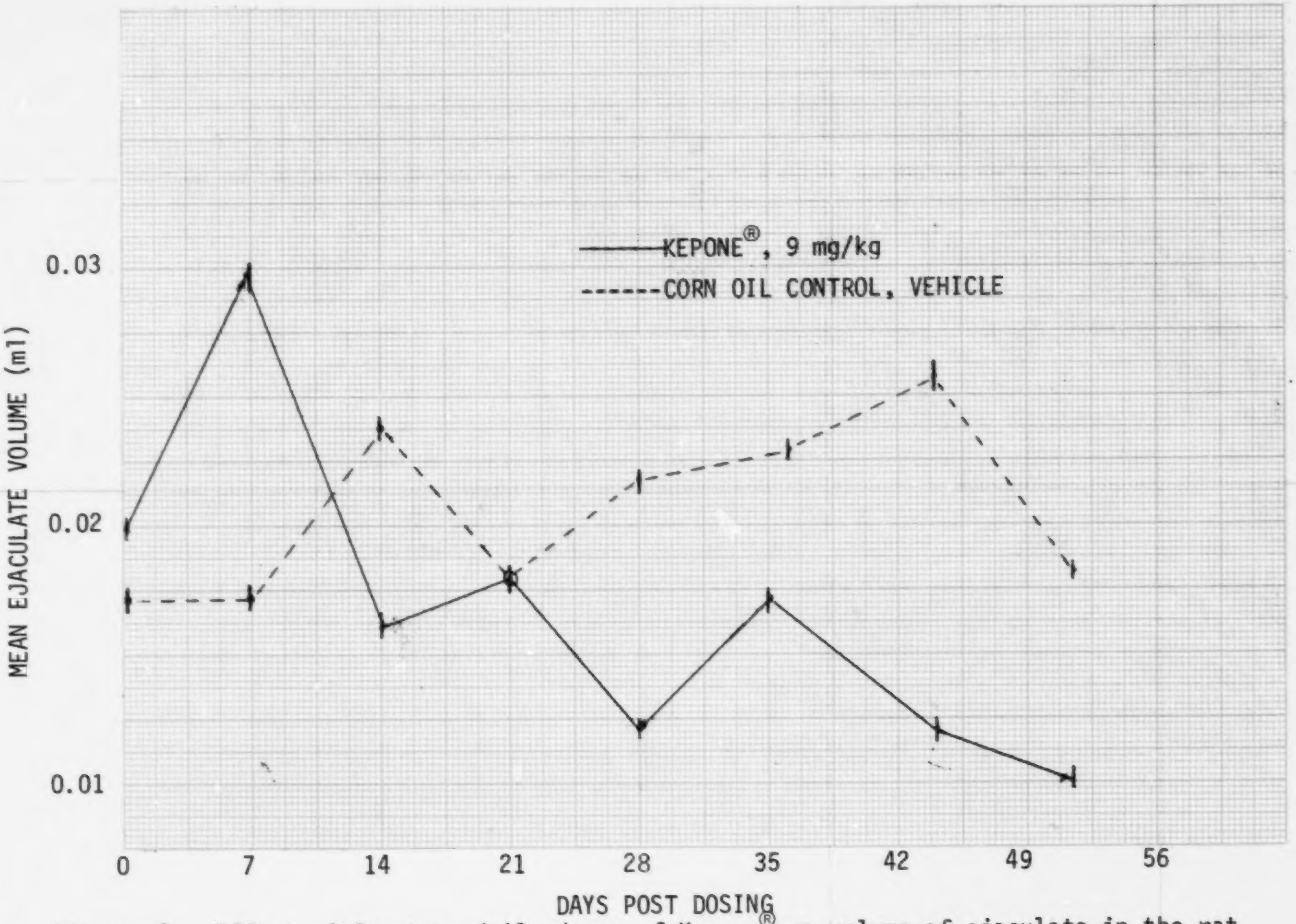

Figure 6. Effect of fourteen daily doses of Kepone® on volume of ejaculate in the rat. N=6. Vertical bars indicate SEM.

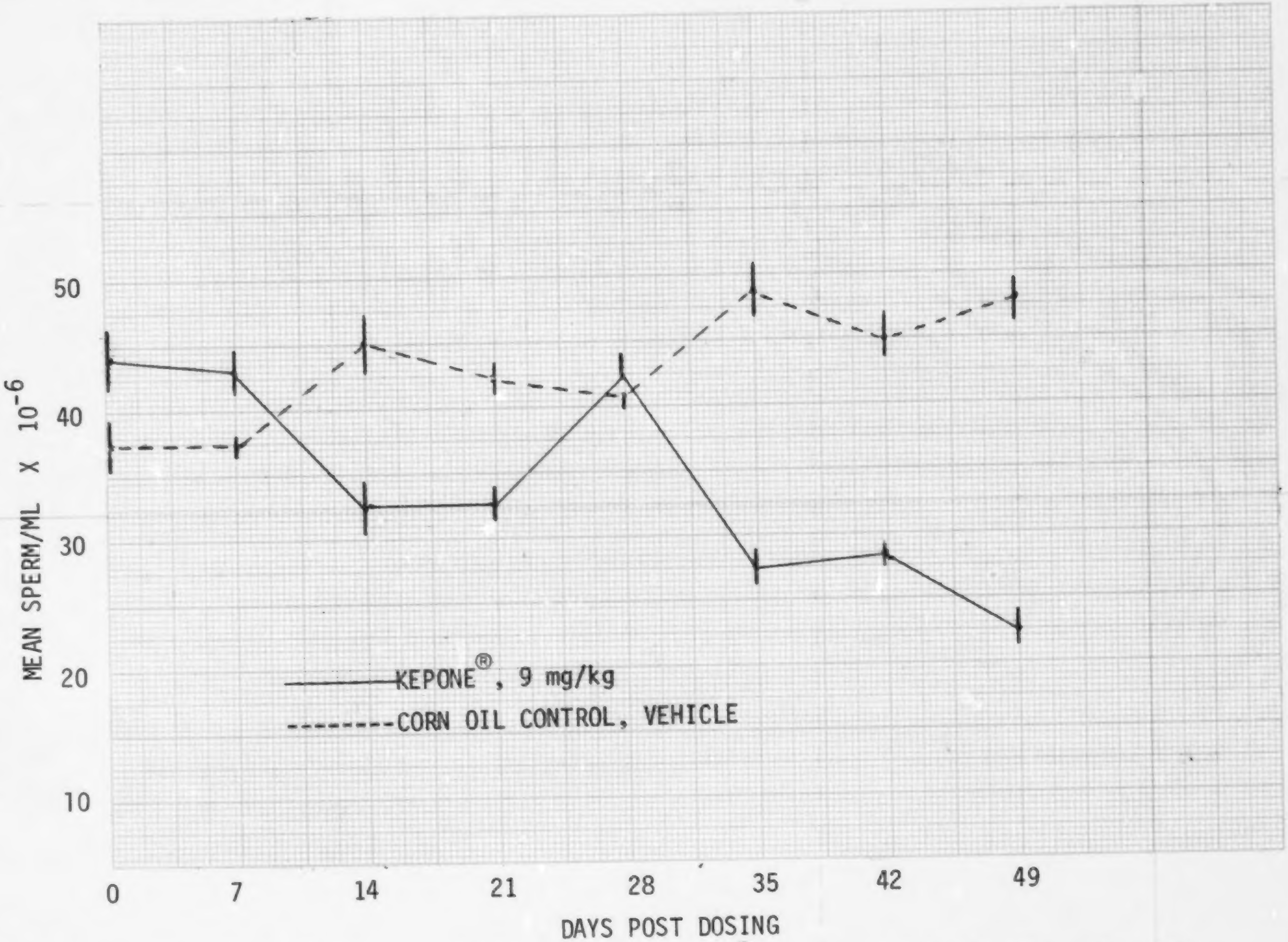

Figure 7. Effect of fourteen daily doses of Kepone® on sperm count in the rat. N=6. Vertical bars indicate SEM.

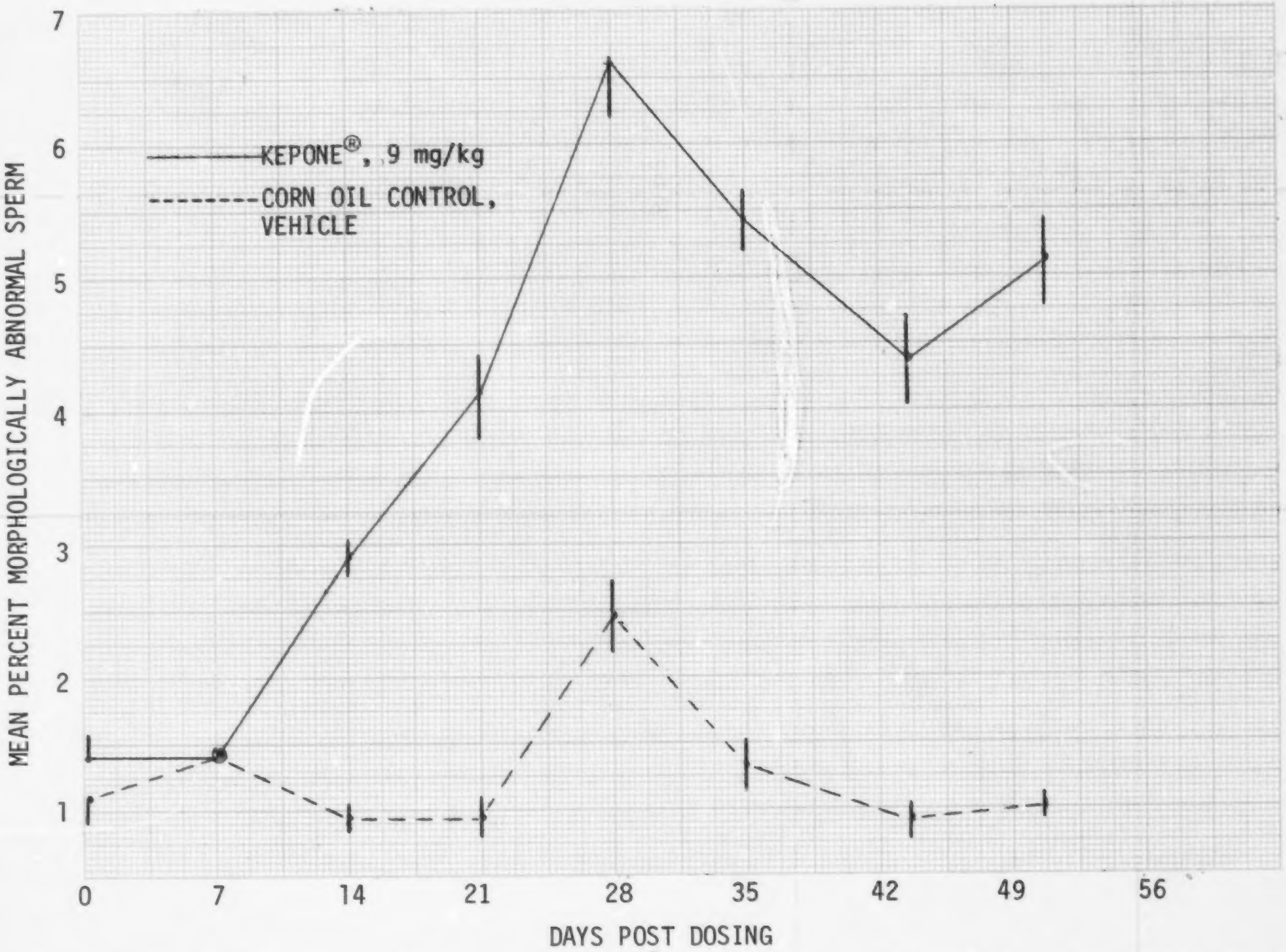

Figure 8. Effect of fourteen daily doses of Kepone® on percent morphologically abnormal sperm in the rat. N=6. Vertical bars indicate SEM.

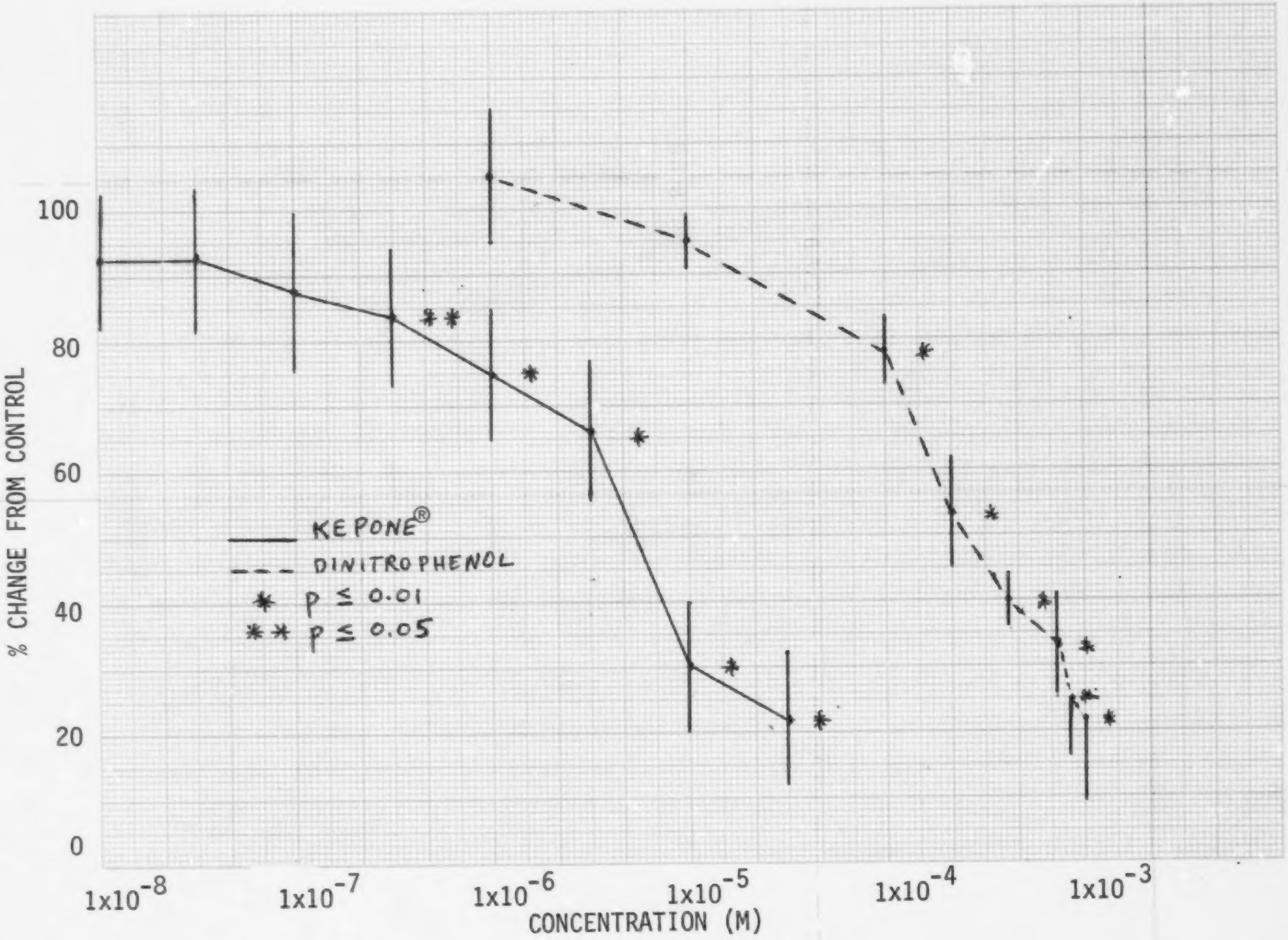

Figure 9. Effect of various concentrations of Kepone® and dinitrophenol on the proliferation of $P388D_1$ cells. N=5. Vertical bars indicate SEM.
Taken From: Carmines et al., 1979.

Our preliminary experiments and the literature suggested that Kepone® might affect energy production. The effect of Kepone® and DNP on oxygen consumption by the $P388D_1$ cells was determined. Figure 10 illustrates the dose-dependent nature of the stimulatory action of cellular respiration of both agents. Solubility limited increasing the concentration of both agents. The time-dependent nature of observed effects was evaluated. Kepone® produced an initial (within 3 min) stimulation of oxygen consumption (Figure 11). The stimulation increased with time (Figure 11) to approximately 250% vehicle control values. The stimulation produced by DNP was found to be time independent (data not shown).

Kepone® may affect calcium metabolism (Couch et al., 1977; Chernoff and Rogers, 1976; Erben, 1972). In addition, Glagoleva et al. (1970) have demonstrated that uncouplers of oxidative phosphorylation can alter the metabolism of this cation. The effect of Kepone® on the kinetics of calcium distribution in the $P388D_1$ was therefore determined. Table 7 summarizes the results of this experiment. Kepone® produced a significant decrease in the size of the mitochondrial exchangeable calcium pool and a significant increase in the efflux rate constant of radiocalcium from this organelle. Kepone® also increased the size of the cytoplasmic pool, but this effect was not statistically significant.

The binding of Kepone® to the cells was determined in order to assess the amount of the pesticide responsible for the effects observed. Figure 12 illustrates the binding of various concentrations of ^{14}C-Kepone® as a function of time. At 60 min 1% of the Kepone® was bound to the cells.

Since the $P388D_1$ cell possesses several macrophage properties, the ability of Kepone® to alter one such property, phagocytosis was investigated. Figure 13 shows the effects of various concentrations of Kepone® on the ability of the $P388D_1$ cell to phagocytize opsonized sheep red blood cells. At the highest concentration (1×10^{-5} M) an initial stimulation followed by a depression in phagocytic activity was observed. At 3×10^{-6} and 1×10^{-6} M, an initial stimulation was observed with values returning to control as the experiment progressed.

Desaiah et al. (1975, 1977a,b) and Folmar (1978) utilizing isolated mitochondrial enzymes have previously demonstrated that Kepone® interferes with energy production. Data presented in this report confirm this observation in isolated cells. Kepone® stimulated oxygen consumption in a manner similar to the classical uncoupler of oxidative phosphorylation, DNP. Kepone® was approximately 80 times more potent than DNP in inhibiting cellular proliferation and stimulating oxygen consumption, suggesting that the mitochondria may be the ultimate site of subcellular toxicity.

The primary function of the mitochondria is that of energy production; secondary to this function is the ability of this organelle to accumulate calcium against a gradient in a process energetically coupled to electron transport. It has been well documented that agents which uncouple or inhibit oxidative phosphorylation or inhibit electron transport disrupt mitochondrial calcium distribution (Rubin, 1974). Data presented in this report demonstrate that Kepone® produced a significant alteration in the

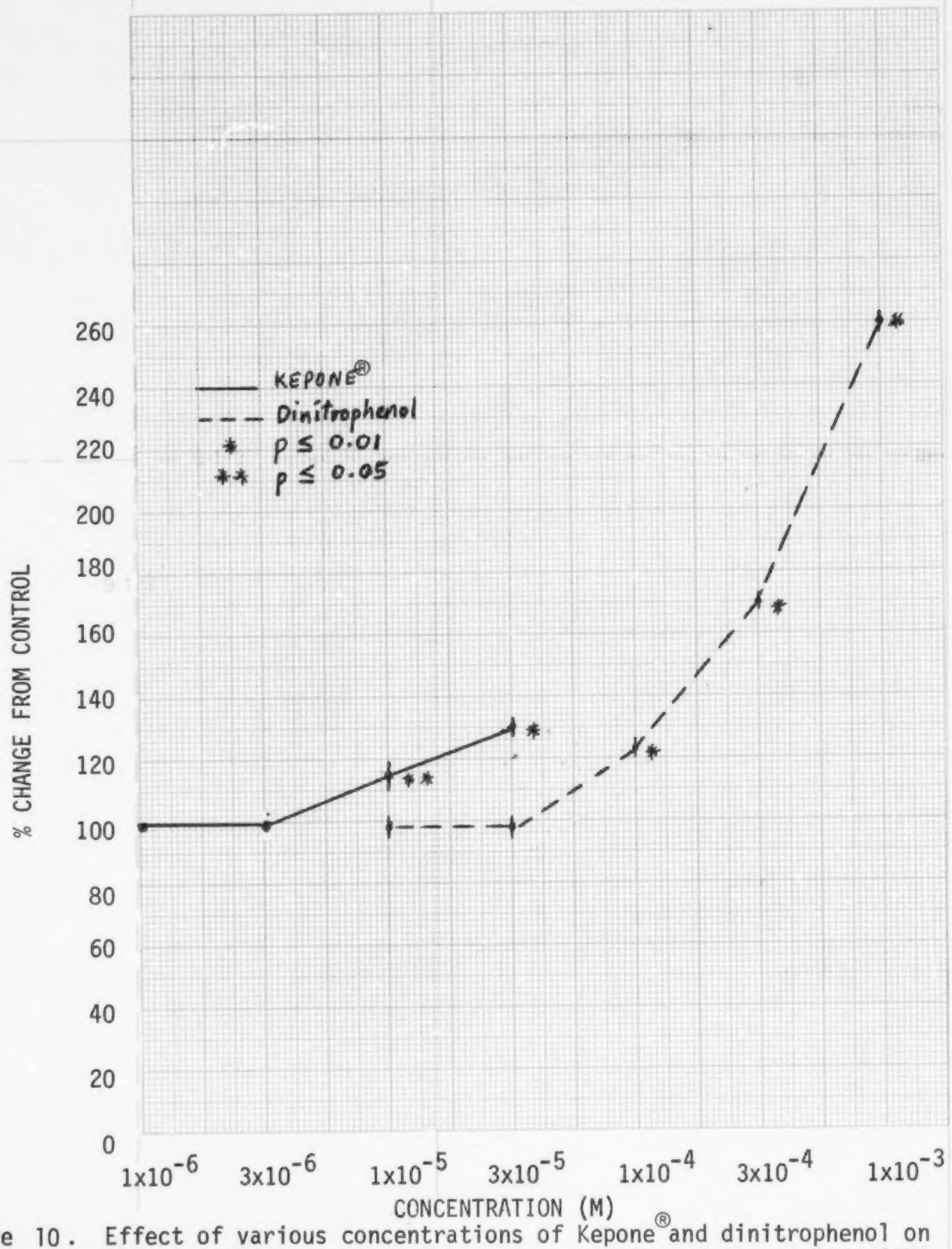

Figure 10. Effect of various concentrations of Kepone® and dinitrophenol on oxygen consumption by $P388D_1$ cells. Three minute preincubation period with both agents. N=4. Vertical bars indicate SEM.
Taken From: Carmines et al., 1979.

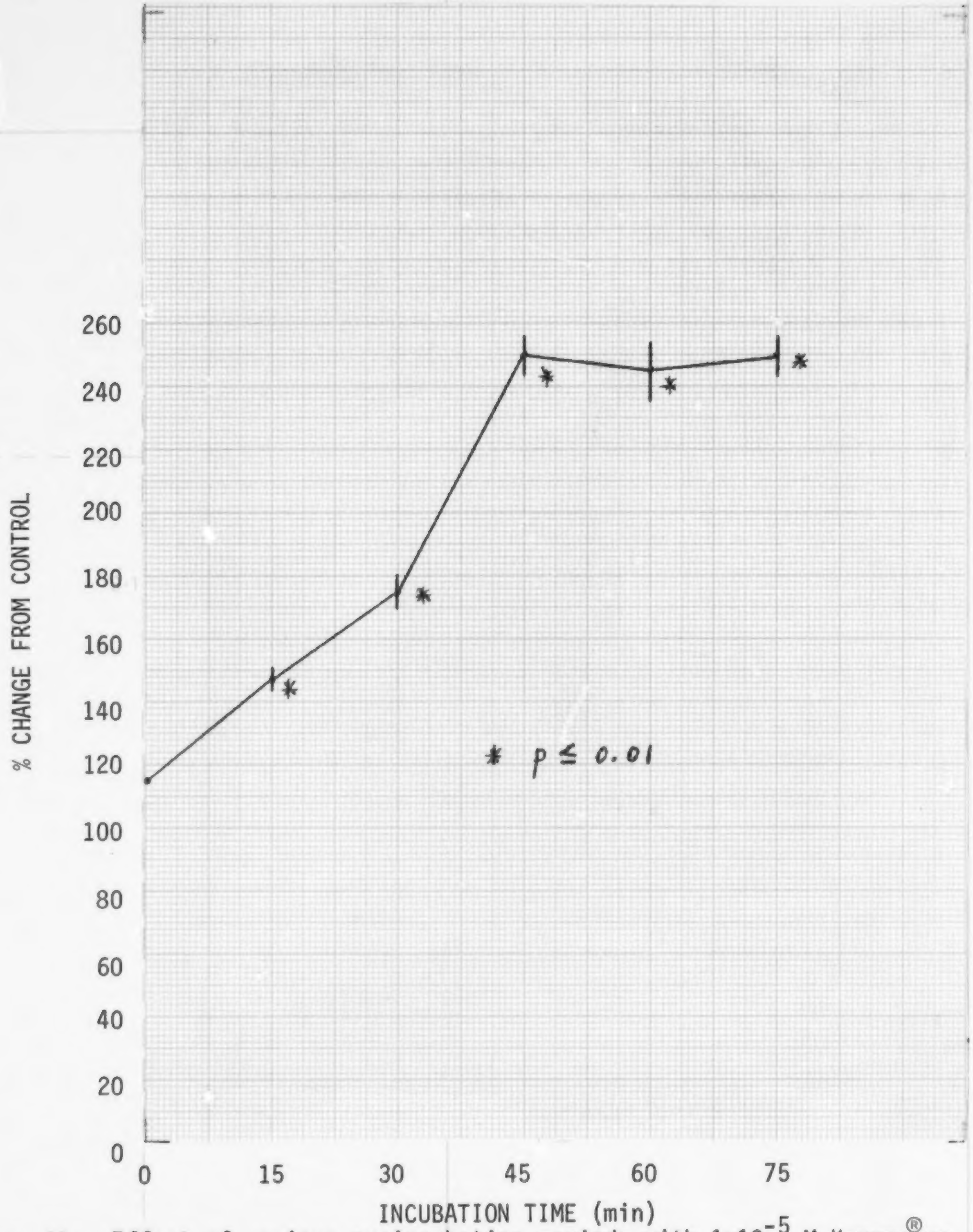

Figure 11. Effect of various preincubation periods with $1x10^{-5}$ M Kepone® on oxygen consumption by the $P388D_1$ cells. N=5. Vertical bars indicate SEM. Taken From: Carmines et al., 1979.

TABLE 7. EFFECT OF KEPONE®(1×10^{-6}M) ON THE DISTRIBUTION AND KINETICS OF RADIOCALCIUM IN THE P388D_1 CELLS

	CYTOPLASM		MITOCHONDRIA	
	Vehicle	1×10^{-6}M Kepone®	Vehicle	1×10^{-6}M Kepone®
Pool size (μm/10^6 cells)	$1.18 \times 10^{-3} \pm 0.37$*	$1.45 \times 10^{-3} \pm 0.15$ 123% (NS)	$8.97 \times 10^{-4} \pm 0.71$	$7.30 \times 10^{-4} \pm 0.61$ 81% ($p \leq 0.05$)§
Rate constant ($\min^{-1}$)	$2.55 \times 10^{-2} \pm 0.14$	$2.45 \times 10^{-2} \pm 0.12$ 96% (NS)†	$7.59 \times 10^{-3} \pm 0.93$	$9.71 \times 10^{-3} \pm 0.52$ 128% ($p \leq 0.05$)§

* N = 4. Values are means ± SEM.

† Not significant.

§ Significant deviation from control values (Student's t-test)

Taken from: Carmines et al., 1979.

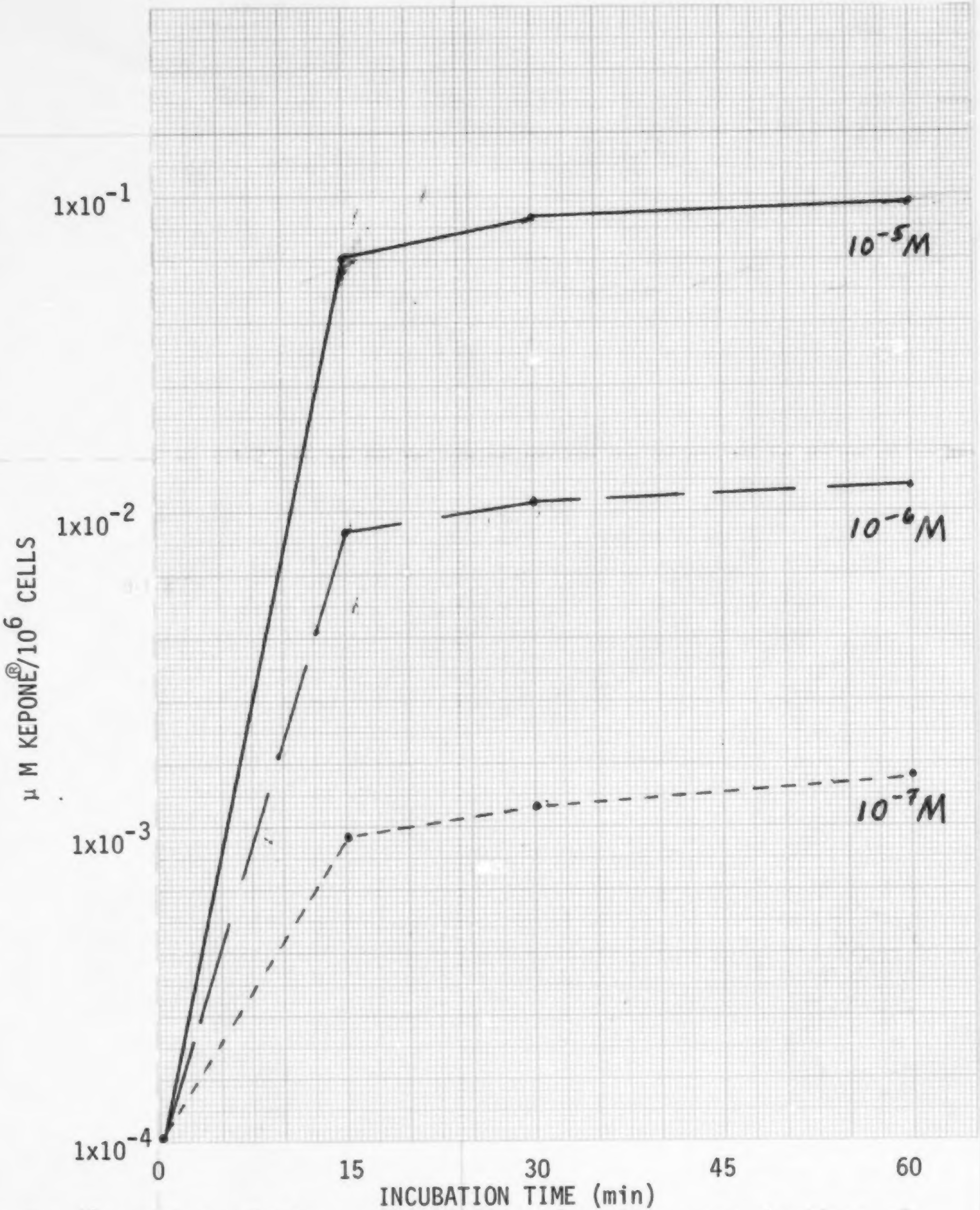

Figure 12. Effect of time on the binding of various concentrations of Kepone® to the P388D$_1$ cells. N=4; mean.
Taken From: Carmines et al., 1979.

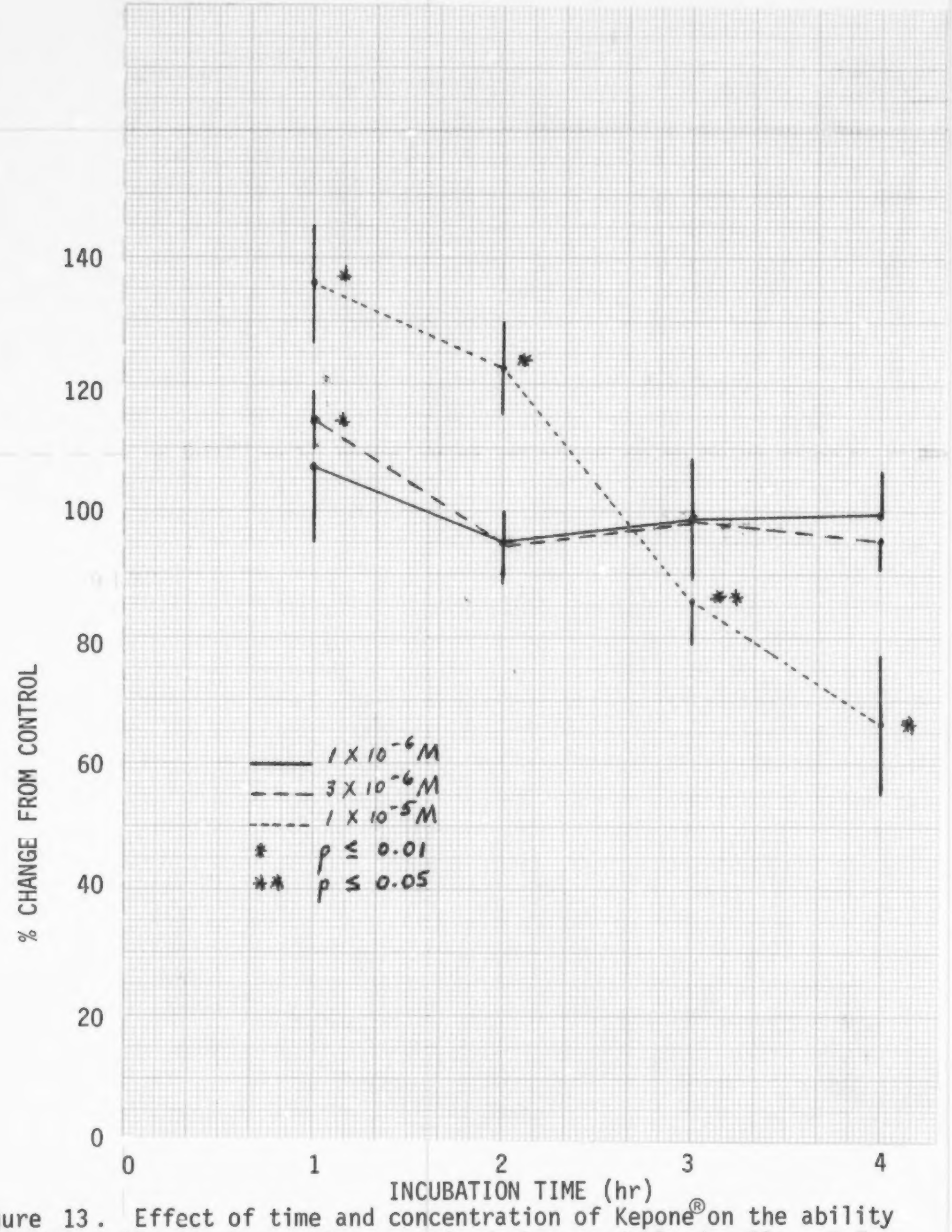

Figure 13. Effect of time and concentration of Kepone® on the ability of P388D$_1$ cells to phagocytize opsonized sheep red blood cells. N=5. Vertical bars indicate SEM.
Taken From: Carmines et al., 1979.

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mitochondrial calcium distribution. These results support the suggestion of Couch et al. (1977) that the syndrome of Kepone® poisoning in fish and in humans may be due to a systemic defect in calcium metabolism.

The significance of Kepone®'s effect on energy metabolism and calcium distribution is revealed in its alteration of the phagocytic process. Previous work in our laboratory (Goodell et al., 1978) has demonstrated the energy-dependent nature of the phagocytic process in the $P388D_1$ cell. It was found that agents which inhibited energy production (i.e., incubation at 4 C) also inhibited the phagocytic process. Kessler and Carchman (submitted) utilizing several agents which alter calcium metabolism, demonstrated that the calcium ion is responsible for stimulation of the phagocytic process. Thus, it appears that our observations of Kepone®'s effects on phagocytic activity (initial stimulation following by inhibition) may be reconciled by the compound's effects on oxygen consumption and calcium metabolism. The initial stimulation of phagocytosis produced by the higher doses of Kepone® may be primarily due to an alteration of calcium metabolism and an inhibition of energy production. The inhibition observed at the end of the experiment, however, may be due to the depletion of energy stores.

Although Kepone®'s in vivo toxicity does not appear to manifest itself as an alteration of the phagocytic process, it is believed that the biochemical alterations which were observed may be useful in understanding its mechanism of toxicity. Calcium has been shown to play a significant role in muscle contraction and neurotransmitter release (Rubin, 1974). Glagoleva et al. (1970) found that uncouplers of oxidative phosphorylation, by interfering with mitochondrial energy production, produced a release of mitochondrial calcium, leading to an increase in neurotransmitter release. The clinical syndrome in humans suggests that the nervous system is the primary target for Kepone® toxicity (Martinez et al., 1977). Reiter et al. (1977) reported that Kepone® treatment in rats leads to hyperreactivity. End et al. (1979) have reported that Kepone® blocks the uptake of calcium into rat brain mitochondria and synaptosomes and stimulates neurotransmitter release. This supports the results obtained from our studies on calcium metabolism. It appears that the effect of Kepone® on isolated cells in culture (alteration of energy metabolism and cellular calcium distribution) may be responsible at least in part for the observed in vivo toxicity.

Taken From: Carmines et al., 1979.

HEXACHLOROBENZENE (HCB)

Dominant Lethal Study

No significant differences among body weights of male rats administered corn oil, HCB, or TEM were observed.

TEM elicited classical dominant lethal mutations during the first 7 weeks of the experimental period (Table 8) (Bateman and Epstein, 1971).

TABLE 8. REPRODUCTIVE DATA FOR FEMALE RATS MATED TO MALES TREATED WITH HEXACHLOROBENZENE
CORN OIL CONTROL (10 mg/kg/day)*

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	14	70	93	12.85	10.85	2.00	10.28	0.57	7/134	5/9	2/12
2	16	80	100	15.81	11.37	4.43	10.81	0.62	10/173	9/7	1/15
3	17	85	100	15.47	12.17	3.29	11.17	1.00	17/190	8/9	2/15
4	13	65	93	15.38	11.61	3.84	11.07	0.53	7/144	6/7	1/12
5	17	85	100	15.00	11.23	3.82	10.88	0.35	6/185	5/12	1/16
6	15	75	100	13.93	10.46	3.46	10.13	0.33	5/152	3/12	1/14
7	18	90	100	15.94	12.22	3.72	11.66	0.55	10/210	6/12	3/15
8	17	85	100	15.52	10.76	4.76	10.35	0.41	7/163	5/12	2/15
9	15	75	100	13.73	10.46	3.26	9.86	0.66	10/148	7/8	3/12
10	16	80	89	10.56	9.18	1.37	8.87	0.31	5/155	4/12	3/13
11	16	80	94	13.87	10.31	3.56	9.68	0.66	10/155	4/12	3/13
12	13	65	100	13.84	11.92	1.92	11.46	0.46	6/49	5/8	1/12
13	15	75	94	15.46	11.00	4.46	10.53	0.46	6/158	6/9	1/14
14	13	65	100	15.92	10.69	5.46	9.53	1.15	15/124	9/4	8/9

* Compound administered for five consecutive days via daily oral gavage.

(continued)

TABLE 8. (Continued)
TEM (0.5 mg/kg)†

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	10	50	100	13.10	11.00	2.10	6.50§	4.50§	20/68¶	10/0¶	10/0¶
2	13	65	93	16.84	10.69	6.15	6.92§	3.76§	49/90¶	12/1¶	10/3¶
3	15	75	94	15.80	10.20§	5.60	4.26§	5.93§	89/64¶	15/0¶	14/1¶
4	17	85	94	15.88	11.47	5.05	8.52	2.35	40/45¶	11/6	7/10
5	15	75	94	15.40	9.66	5.73	8.93	0.73	11/134	8/7	2/13
6	11	55	100	13.81	10.81	3.36	9.27	1.54	17/102¶	4/7	1/10
7	17	85	100	15.47	10.35§	5.17	9.17§	1.17	20/156¶	8/9	5/12
8	13	65	86	15.61	10.92	4.76	10.15	0.76	10/132	7/6	2/11
9	16	80	94	13.50	8.43§	5.06	7.93	0.60	8/127	7/9	1/15
10	13	65	81	11.23	10.15	1.69	9.46	0.69	9/123	4/9	2/11
11	16	80	94	16.37	11.06	5.31	10.50	0.50	8/169	7/9	1/15
12	11	55	79	17.27	11.00	6.27§	10.54	0.45	5/116	4/7	1/10
13	17	85	100	15.52	10.64	4.88	9.52	1.00	17/162	10/7	2/15
14	16	80	94	14.75	9.62	5.12	9.31	0.31§	5/149¶	5/11	0/16

† Compound administered as a single dose via oral gavage.

§ Significantly different from control (Student's T-test, $p \leq 0.05$).

¶ Significantly different from control (χ^2 with Yates' correction $p \leq 0.05$).

(Continued)

TABLE 8. (Continued)
HCB* (70 mg/kg/day)

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	10	50	83	15.60	11.00	4.60	10.80	0.20	2/108	2/8	0/10
2	14	70	93	14.71	10.78	4.57	10.00	0.85	12/140	8/6	1/12
3	16	80	94	13.68	11.93	1.87	11.31	0.62	10/181	8/8	1/15
4	17	85	93	17.46	10.46	7.00	9.92	0.53	7/129	5/8	2/11
5	13	65	87	14.23	11.23	3.07	10.84	0.38	5/141	4/9	1/12
6	17	85	100	16.11	10.17	5.94	9.58	0.58	10/163	8/9	2/15
7	13	65	100	16.76	11.92	4.84	11.38	0.53	7/148	7/6	0/13
8	15	75	100	15.40	10.66	4.73	9.66	1.00	14/145	8/7	3/12
9	15	75	94	14.26	10.40	3.86	9.86	0.66	10/148	4/11	3/12
10	17	85	100	10.94	10.00	1.00	9.52	0.47	8/162	5/12	2/15
11	15	75	79	16.60	11.00	5.60	10.53	0.46	7/158	5/10	1/14
12	15	75	100	15.20	10.80	4.40	9.60	0.53	8/144	7/8	1/14
13	14	70	93	12.42§	10.35	2.14§	9.78	0.57	8/137	5/9	2/12
14	13	65	93	15.61	10.23	5.38	8.84	0.69	9/124	7/6	2/11

* Compound administered for five consecutive days via daily oral gavage.

§ Significantly different from control (Student's T-test, $p \leq 0.05$).

(Continued)

TABLE 8. (Continued)
HCB* (221 mg/kg/day)

Week	Number Pregnant	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≧1	DF≧2
1	9¶	45	100	13.00	11.66	1.44	10.88	0.77	7/88	3/6	2/7
2	8¶	40	100	12.50	10.25	1.75	9.75	0.62	4/78	3/5	1/7
3	11¶	55	100	14.90	11.63	3.27	10.45	1.18	13/113	8/3	2/9
4	10	50	100	14.49	10.30	4.10	10.10	0.20	2/101	2/8	0/10
5	12	60	92	16.41	11.08	5.33	10.66	0.41	5/128	4/8	1/11
6	16	80	100	14.37	10.37	4.00	10.00	0.37	6/160	6/10	0/16
7	11¶	55	73	16.41	11.54	4.90	11.00	0.54	6/121	6/5	0/11
8	15	75	100	14.06	10.33	3.73	9.60	0.73	11/144	9/6	2/13
9	16	80	100	15.25	8.12	4.62	10.06	0.56	9/161	7/9	2/14
10	12	60	75	14.08§	11.66§	2.41	10.91	0.75	9/131	6/6	1/11
11	13	65	93	13.38	9.69	3.69	9.23	0.46	6/120	5/8	11/12
12	10	50	91	12.40	9.20§	3.20	8.80§	0.40	4/88	3/7	1/9
13	11	55	85	14.81	10.81	4.09	9.72	1.09§	12/107	8/3	4/7
14	12	60	100	14.33	10.66	3.66	10.58	0.08§	1/127¶	1/11¶	0/12

† Compound administered for five consecutive days via daily oral gavage.

§ Significantly different from control (Student's T-test, $p \leqq 0.05$).

¶ Significantly different from control (χ^2 with Yates' correction $p \leqq 0.05$).

Taken from: Simon _et_ _al_., 1979

The female rats mated to males that received HCB showed the following statistically significant events. There was a decrease in corpora lutea and preimplantation losses during Week 13 at the low dose. At the high dose, there was an increase in corpora lutea, implantations, and living fetuses during Week 10; a decrease in implantations and living fetuses during Week 12; an increase in resorptions during Week 13; and a decrease in resorptions, the proportion of nonliving to living fetuses, and the proportion of females bearing one or more resorptions during Week 14.

HCB did not induce any classical dominant lethal events during the early weeks (1-5) of mating (Bateman, 1966). Statistically significant effects among the HCB-treated groups were observed during weeks 10-14. These effects were inconsistent in their extent and direction. Animals treated with HCB did not perform as well as controls during Weeks 12 and 13, but performed better than controls during Weeks 10 and 14. When compared with 14 weeks of pooled controls, no significant effects were seen among the HCB-treated groups. These inconsistent results may be attributed to weekly fluctuations in the test groups.

HCB apparently had a dose-dependent effect upon male reproductive performance. At the low dose, the males impregnated fewer females (vs control males) in 8 of the 14 weeks and, at the high dose, 12 of the 14 weeks (Mating Index). The effect at the high dose was highly significant ($p < 0.001$) using the sum of χ^2 procedure (Mantel and Haenszel, 1959). The percentage of inseminated females made pregnant (Fertility Index) was not adversely affected.

HCB is well absorbed from the gastrointestinal tract (Mehendale *et al*., 1975) and persists in rat tissues for months following 33 weeks *of* dietary administration (Borzelleca *et al*., 1971). In the studies described herein, the administration of up to 221 mg/kg HCB for 5 days failed to elicit dominant lethal effects, but adversely affected male reproductive performance up to 14 weeks after HCB administration.

Khera (1974) reported a lack of dominant lethal mutations in rats following administration of up to 60 mg/kg HCB for 10 days and reported no effects upon reproduction. Grant *et al*. (1977) reported decreased reproductive performance by males and females exposed to HCB at dietary levels of up to 640 ppm for life. Their experimental conditions were similar to those of Khera with three exceptions: (1) both males and females were fed HCB; (2) Khera's rats were exposed by gavage; and (3) exposure commenced during the weanling stage of life, and continued through the next three generations. These adverse effects reported to be due to HCB might be the result of increased sensitivity to HCB during the early stages of life. The work of Kimbrough and Linder (1974) and Grant *et al*. (1974) suggests that female rats may be more sensitive than males to some of the effects of HCB (respiratory, porphyria) but equally or less sensitive to others. The effects seen by Grant *et al*. (1977) might be explained by this differential sensitivity, though specific effects upon the female reproductive system were not reported by Kimbrough and Linder (1974).

It is concluded that HCB is not a dominant lethal mutagen under the conditions tested, but can have adverse effects upon male reproductive performance.

Taken From: Simon et al., 1979.

Effects on Sperm Morphology

Rats which received daily oral gavage doses (35 mg/kg) of HCB in corn oil for 14 days showed no significant differences from the corn oil control animals when the following parameters were examined: body weight, ejaculate volume, sperm count, and percent of sperm with abnormal morphology. See Figures 14-17.

These results are consistent with the findings that HCB fails to elicit dominant lethal mutations in the rat.

DIBROMOCHLOROMETHANE (DBCM) AND TRICHLOROMETHANE (TCM)

Modified Multigeneration Reproduction Study

General Observations --

Ingestion of TCM and DBCM produced a significant decrease in body weight gain in the high concentration treated (5.0 and 4.0 mg/ml, respectively) animals of both the F/0 and F/1b generations (Table 9). This decrease was not due to a taste aversion since fluid consumption did not appear to be altered. Survival was reduced in the 5 mg/ml TCM F/0 generation (males and females), all treated males in the F/1b generations, and the high concentration females (both TCM and DBCM) in the F/1b generation, and the high concentration females (both TCM and DBCM) in the F/1b generation (Table 10). Upon necropsy (Table 11) almost all animals in the high dose groups of both generations were observed to have enlarged livers with morphology characteristic of hepatotoxicity. This measure of toxicity appeared to be dose-dependent.

Fertility --

Fertility was significantly reduced among mice ingesting 5 mg/ml TCM and 4 mg/ml DBCM (Table 12) in the F/1a, F/1b, F/2a, and F/2b (TCM) and F/1c and F/2a (DBCM) generations. The mating index (MI) for the F/1c vehicle control was 0.43. An explanation for this observation was not apparent. The vehicle controls appeared healthier than the naive or treatment groups. When statistical tests were performed on the F/1c generation (MI), all of the DBCM treatments and the 0.1 mg/ml TCM treatments were significantly different. When the MI for all generations was used as a control, these values were not significantly different. (The 5.0 mg/ml TCM was statistically different from the control group.) Generally, throughout the study, the MI decreased with increasing concentrations of test material and decreased with the progression of different generations.

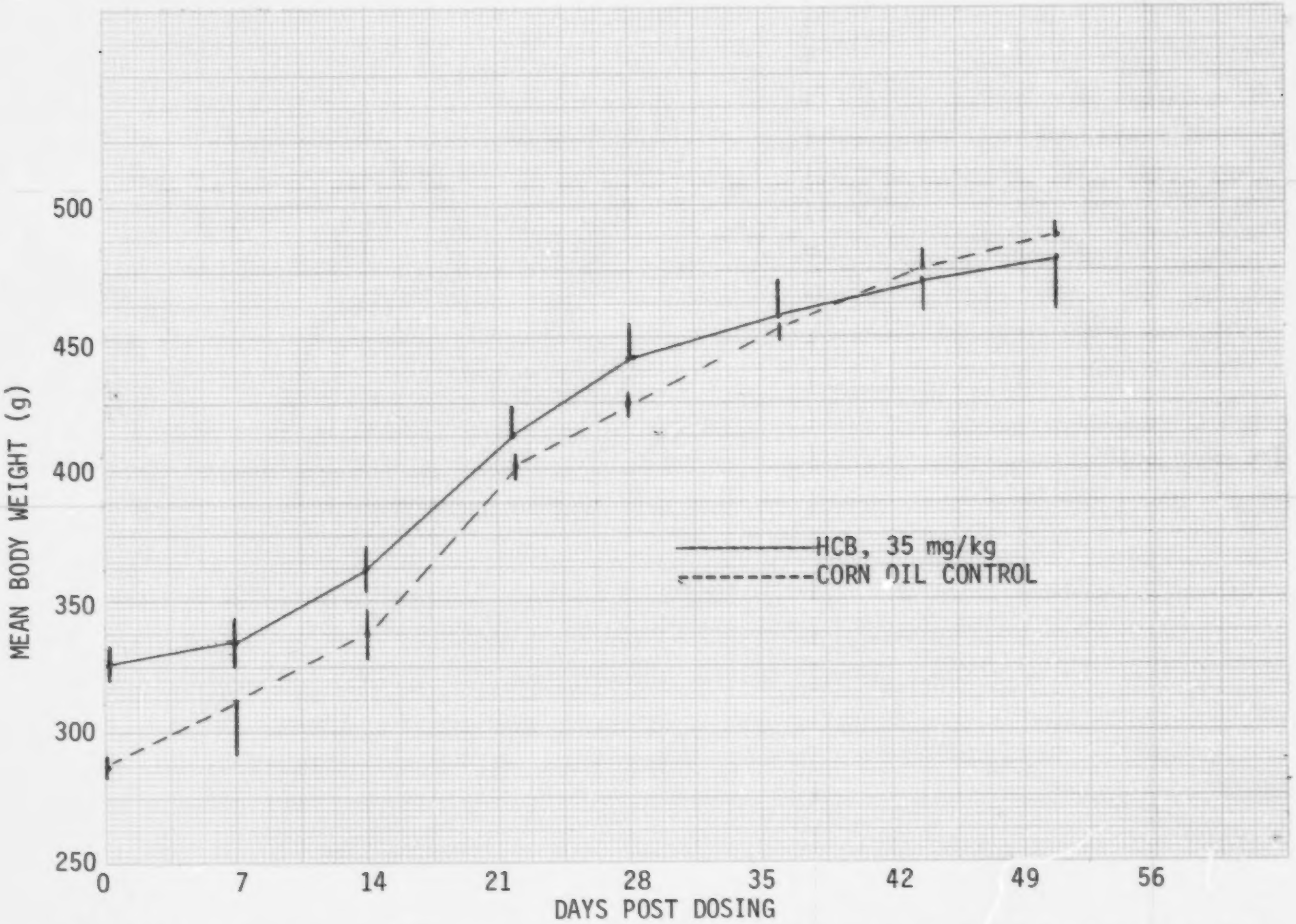

Figure 14. Effect of fourteen daily doses of HCB on male rat body weight. N=6. Vertical bars indicate SEM.

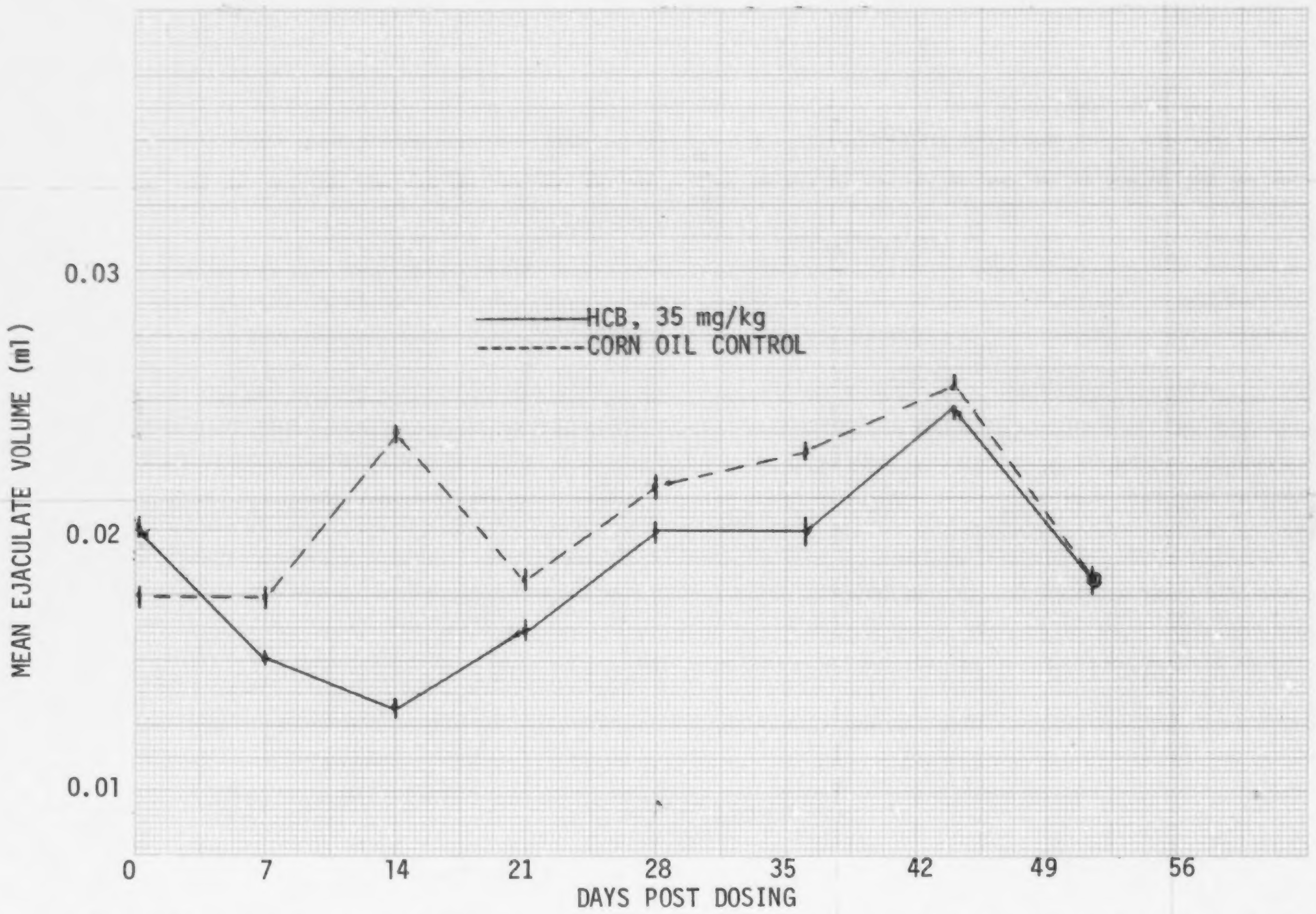

Figure 15. Effect of fourteen daily doses of HCB on volume of ejaculate in the rat. N=6. Vertical bars indicate SEM.

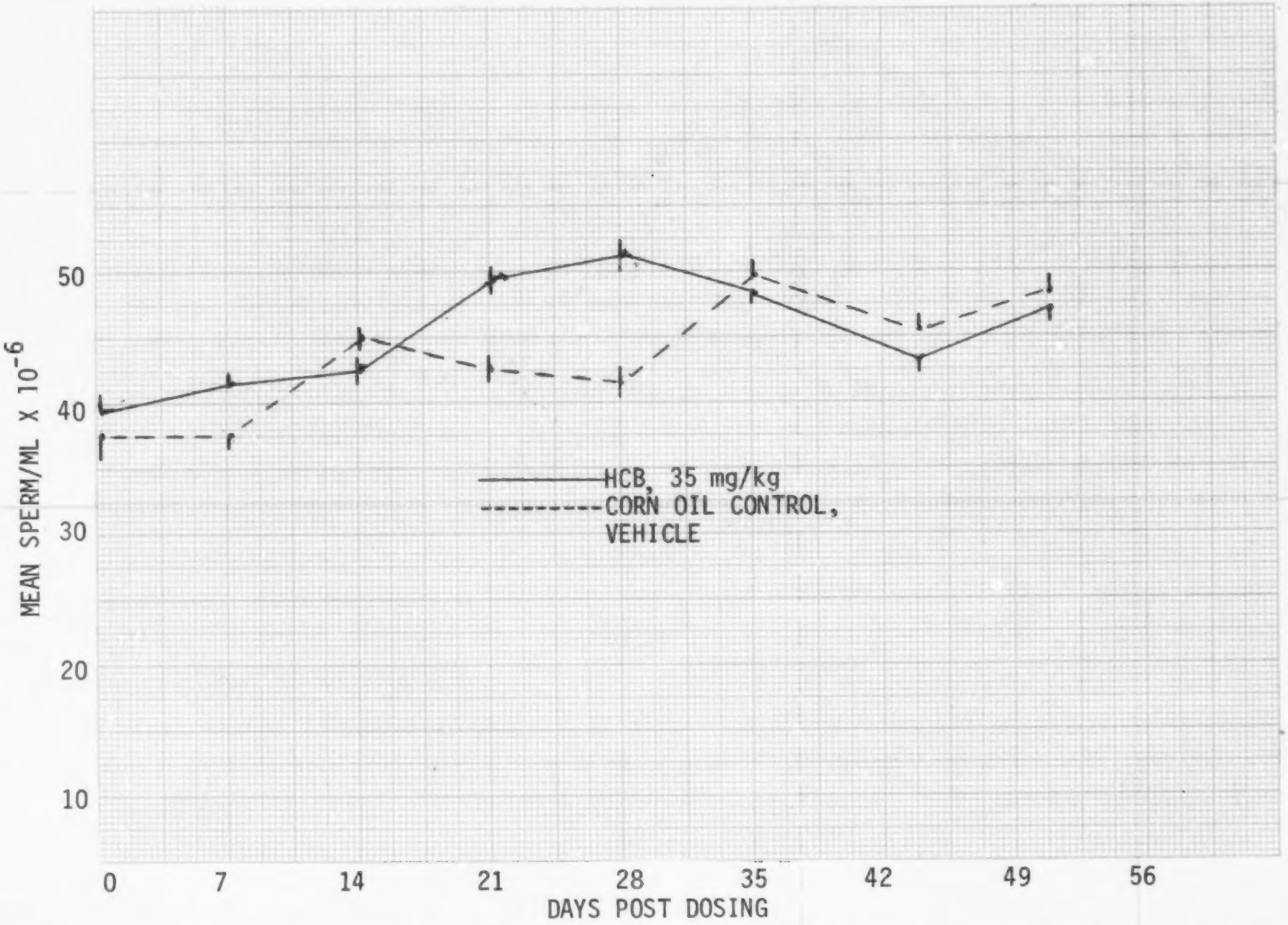

Figure 16. Effect of fourteen daily doses of HCB on sperm count in the rat. N=6. Vertical bars indicate SEM.

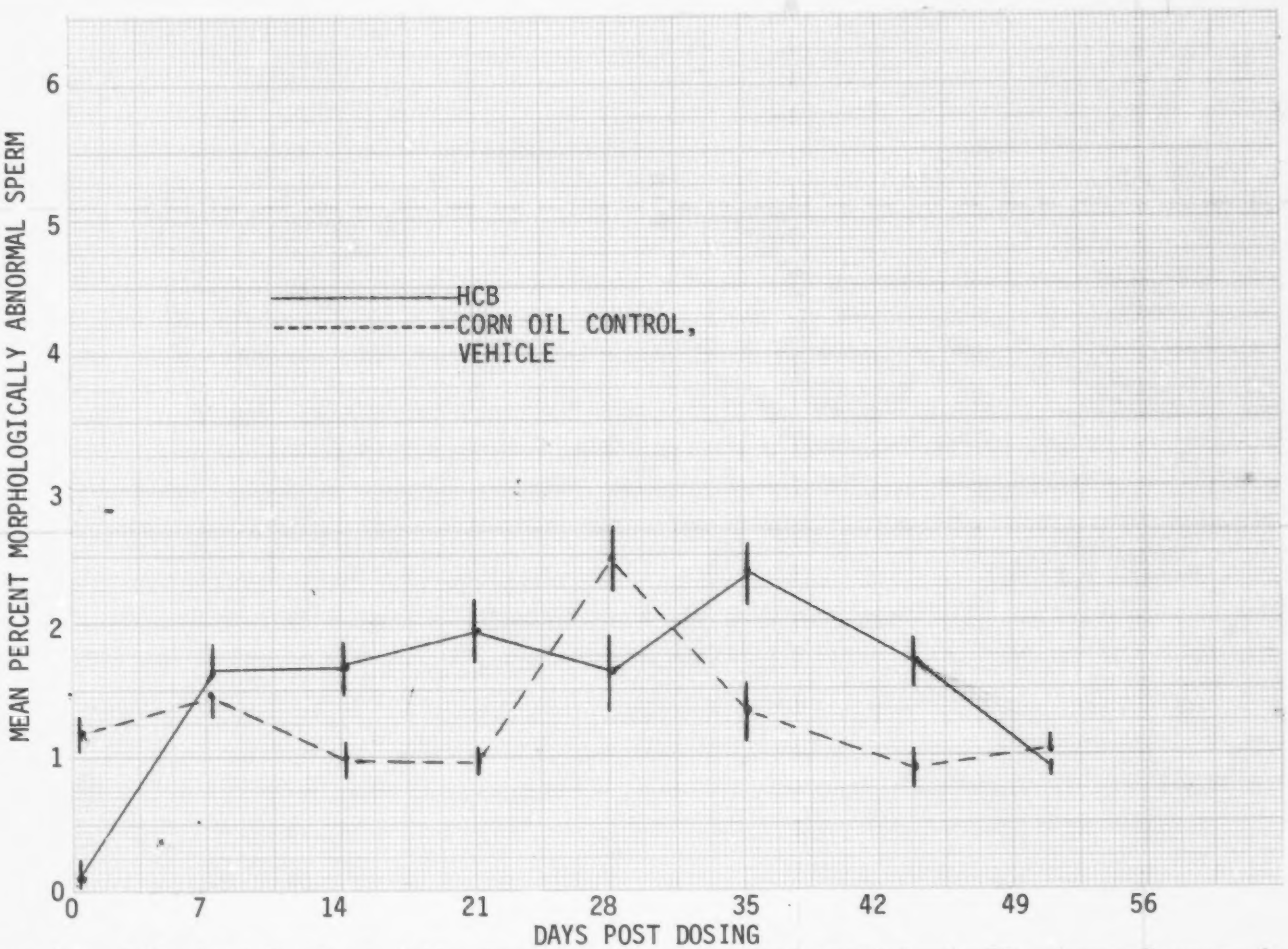

Figure 17. Effect of fourteen daily doses of HCB on percent morphologically abnormal sperm in the rat. N=6. Vertical bars indicate SEM.

TABLE 9
AVERAGE TERMINAL BODY WEIGHT OF MICE INGESTING DBCM AND TCM

Group	Concentration mg/ml	F/0		F/1b	
		Males	Females	Males	Females
Control	0.0 (Naive)	40.86*	44.45	38.34	45.30
	0.0 (Naive)	38.36	45.27	37.22	42.54
	0.0 (Vehicle)†	40.12	46.99	38.85	44.76
DBCM	0.1	37.72	48.25	40.11	46.58
	1.0	38.96	43.16§	38.56	39.77§
	4.0	35.33§	37.66§	30.01§	35.54§
TCM	0.1	40.32	47.90	38.85	46.65
	1.0	38.32	46.48	37.85	41.43§
	5.0	32.45§	39.42§	33.69§	34.72§

* in grams

† Vehicle = Emulphor:water (1:1000)

§ Statistically different from vehicle control when analyzed for change in average body wieght (Kruskal Wallis χ^2 approximation with Dunn's approximation to a distribution free multiple comparison).

Taken from: Riddle et al., in press.

TABLE 10. SURVIVAL OF MICE INGESTING DBCM AND TCM*

Group	Concentration mg/ml	F/0		F/1b	
		Males	Females	Males	Females
Control	0.0 (Naive)	10	30	8	29
	0.0 (Naive)	10	28	5†	21
	0.0 (Vehicle)§	10	29	5	27
DBCM	0.1	9†	26	4	29
	1.0	10	29	6	28
	4.0	10	29	0¶	15
TCM	0.1	9	27	4	24
	1.0	10	28	2	26
	5.0	6	18	1	21

* Except where indicated, all groups of male mice started treated with 10 males per group, and all groups of female mice started treatment with 30 females per group.

† This group commenced treatment with 9 animals.

§ Vehicle = Emulphor:water (1:1000)

¶ This group commenced treatment with 6 animals.

Taken from: Riddle _et al_., in press.

TABLE 11. SEVERITY OF GROSS LIVER PATHOLOGY OF MICE INGESTING DBCM AND TCM*

Group	Concentration (mg/ml)	N	0	+	++	+++	++++	% with Lesions
				F/0				
Control	0.0 (Naive)	4	100%	0.0%	0.0%	0.0%	0.0%	0%
	0.0 (Vehicle)	15	100%	0.0%	0.0%	0.0%	0.0%	0%
DBCM	0.1	20	75.0%	25.0%	0.0%	0.0%	0.0%	25%
	1.0	23	30.4%	34.8%	30.4%	4.3%	0.0%	70%
	4.0	22	0.0%	18.2%	36.4%	27.3%	18.2%	100%
TCM	0.1	21	85.7%	14.3%	0.0%	0.0%	0.0%	14%
	1.0	21	0.0%	61.9%	28.6%	9.5%	0.0%	100%
	5.0	12	8.3%	16.7%	16.7%	25.0%	33.3%	92%
				F/1b				
Control	0.0 (Naive)	67	100%	0.0%	0.0%	0.0%	0.0%	0%
	0.0 (Vehicle)	36	100%	0.0%	0.0%	0.0%	0.0%	0%
DBCM	0.1	39	82.0%	15.4%	2.6%	0.0%	0.0%	18%
	1.0	38	35.9%	44.7%	18.4%	0.0%	0.0%	64%
	4.0	20	0.0%	25.0%	40.0%	20.0%	15.0%	100%
TCM	0.1	32	90.6%	9.4%	0.0%	0.0%	0.0%	9%
	1.0	35	37.1%	54.3%	8.6%	0.0%	0.0%	64%
	5.0	28	0.0%	17.8%	28.6%	21.4%	32.1%	100%

* Grading of Gross Liver Pathology:

0 - Normal color and morphology
+ - Slight yellow-gray color, presumably due to accumulation of fat deposits; morphology normal
++ - More pronounced yellow-gray discoloration; appearance of surface markings, but no distinct masses
+++ - Gray discoloration; distinct masses present on liver surface
++++ - Gray to black discoloration; large nodules (≧ 3 mm in diameter) present on and within liver surface

Taken from: Riddle *et al.*, in press.

TABLE 12. REPRODUCTIVE INDICES BY LITTER OF MICE INGESTING DBCM AND TCM

Group	Concentration (mg/ml)	F/1a		F/1b		F/1c		F/2a		F/2b	
		MI	GI	MI	GI	MI	GI	MI	GI	MI	GI
Control	0.0 (Naive)	1.00	1.00	1.00	1.00	0.75	0.67	0.96	1.00	*	*
	0.0 (Naive)	1.00	0.96	1.00	1.00	†	†	0.89	1.00	*	*
	0.0 (Vehicle)§	1.00	1.00	0.96	1.00	0.43	0.90	1.00	1.00	0.71	0.93
DBCM	0.1	1.00	1.00	1.00	1.00	0.90¶	1.00	0.95	1.00	0.91	0.95
	1.0	1.00	1.00	0.96	1.00	0.83¶	0.84	0.85	1.00	0.86	1.00
	4.0	0.96	0.91¶	0.88	0.78¶	0.70	0.31¶	0.25¶	1.00	1.00	1.00
TCM	0.1	1.00	1.00	1.00	1.00	0.81¶	0.88	0.94	1.00	0.88	1.00
	1.0	0.97	1.00	1.00	1.00	0.77	1.00	0.94	1.00	0.60	1.00
	5.0	0.84¶	0.94¶	0.80¶	0.94	0.58	0.57¶	0.58¶	0.86¶	0.50	0.86

* Animals were used for dominant lethal and teratology studies.

† Animals from both naive treatment groups were combined.

§ Vehicle = Emulphor:water (1:1000)

¶ Statistically different from vehicle control; Kruskal Wallis χ^2 approximation with Dunn's approximation to a distribution-free multiple comparison; $p \leqq 0.05$.

Litter Size at Birth --

Compared to the vehicle control, the average number of pups (live or dead per litter at birth was significantly decreased in all generations ingesting the high concentrations of TCM (5 mg/ml) and DBCM (4 mg/ml) (Table 13). TCM (0.1 and 1.0 mg/ml) and DBCM (0.1 mg/ml) were without effects on litter size in all generations. DBCM (1.0 mg/ml) significantly decreased the number of pups in the F/1c generation with no effects on the other generations. A comparison of the litter size at birth (Table 13) and the mating index (Table 12) indicated a positive correlation. That is, the treatment groups which exhibited a reduction in fertility also exhibited a reduction in litter size.

Gestation Survival --

Among the offspring of mice ingesting the high concentrations of DBCM (4.0 mg/ml) and TCM (5.0 mg/ml), the gestation index (GI; the percentage of litters with pups alive at parturition) was significantly lower than vehicle controls (Table 12). DBCM (4.0 mg/ml) reduced the GI in the F/1a, F/1b, and F/1c generations. TCM (5.0 mg/ml) reduced the GI in the F/1a, F/1c, and F/2a generations. In all cases, a reduction in GI was associated with a decrease in fertility. DBCM and TCM (at 0.1 and 1.0 mg/ ml) were without statistically significant effects on the GI.

Postnatal Survival --

The viability index (VI; the percentage of live born pups surviving 4 days of age) was significantly reduced by both DBCM and TCM (Table 14). DBCM reduced the VI in a concentration-dependent manner in all generations. DBCM (4.0 mg/ml) significantly reduced the VI in the F/1a, F/1b, F/1c, and F/2a generations. DBCM (1.0 mg/ml) reduced the VI only in the F/1b generation. TCM (5.0 mg/ml) decreased the VI in the F/1a, F/1a and F/2a generations. A reduction in the viability of the live born pups (VI) was associated with a concurrent reduction in FI, litter size, and GI. The low concentration (0.1 mg/ml) of DBCM and TCM were without effects on the VI.

The lactation index (LI; the percentage of 4-day-old pups surviving 21 days) was sporadically affected by DBCM and TCM (Table 14). DBCM (1.0 mg/ml) caused a significant reduction in the F/1b and F/2b generations. The high concentration (4.0 mg/ml; DBCM) significantly reduced the LI only in the F/1a generation. At 5.0 mg/ml, TCM significantly altered the LI in the F/1a and F/2b generations. The low concentration (0.1 mg/ml) of DBCM and TCM were without effects on the LI. Generally, a reduction in the VI was not associated with a concurrent reduction in the LI.

Postnatal Body Weight --

The average body weight of the pups of mice ingesting DBCM (Table 15) was significantly reduced only in the F/2a generation. The effect was concentration-dependent, being observed at all treatment levels. DBCM did not significantly alter the body weights in any of the other generations.

TABLE 13
EFFECTS OF PARENTAL INGESTION OF DBCM AND TCM ON THE AVERAGE NUMBER OF PUPS PER LITTER AT BIRTH

Group	Concentration (mg/ml)	F/1a	F/1b	F/1c	F/2a	F/2b
Control	0.0 (Naive)	13	15	*	11	*
	0.0 (Vehicle)	13	14	13	12	12
DBCM	0.1	13	15	11	11	13
	1.0	12	13	9†	11	12
	4.0	10†	8†	8†	8†	8†
TCM	0.1	12	15	13	11	13
	1.0	12	13	12	10	12
	5.0	9†	10†	8†	9†	9†

* These animals were used for the dominant lethal and teratology studies.

† Statistically different from vehicle control values. Kruskal Wallis χ^2 approximation with Dunn's approximation to a distribution-free multiple comparison, $p \leqq 0.05$.

Taken from: Riddle _et al_., in press.

TABLE 14. EFFECTS OF PARENTAL INGESTION OF DBCM AND TCM ON LITTER SURVIVAL INDICES

Group	Concentration (mg/ml)	F/1a		F/1b		F/1c		F/2a		F/2b	
		VI	LI	VI	LI	VI	LI	VI	LI	VI	LI
Control	0.0 (Naive)	0.97	0.88	0.94	0.92			0.93	0.84		
						1.00*	0.92*			0.00†	0.00†
		0.95	0.88	0.92	0.92			0.91	0.93		
	0.0 (Vehicle§)	0.98	0.90	0.97	0.91	0.95	0.94	0.87	0.79	0.90	0.90
DBCM	0.1	0.93	0.81	0.96	0.91	0.97	0.90	0.91	0.91	0.96	0.89
	1.0	0.82	0.78	0.70¶	0.72¶	0.85	0.84	0.78	0.67	0.86	0.68¶
	4.0	0.47¶	0.74	0.44¶	0.88	0.48¶	0.90	0.20¶	1.00	0.71	0.66¶
TCM	0.1	0.94	0.81	0.85	0.87	0.91	0.91	0.93	0.85	0.94	0.92
	1.0	0.81	0.66¶	0.63¶	0.82	0.80	0.80	0.85	0.83	0.88	0.82
	5.0	0.60¶	0.68¶	0.67¶	0.87	0.62	0.88	0.52¶	0.88	0.81	0.60¶

* Animals from both naive treatment groups were combined.

† Animals were used for dominant lethal and teratology studies.

§ Vehicle = Emulphor:water (1:1000)

¶ Statistically different from vehicle control (Kruskal Willis χ^2 approximation with Dunn's approximation to a distribution-free multiple comparison, $p \leq 0.05$).

Taken from: Riddle *et al.*, in press.

TABLE 15
POSTNATAL BODY WEIGHT AMONG OFFSPRING OF MICE INGESTING DBCM

		Concentration (mg/ml)				
Generation	Day	0.0*	0.0†	0.1	1.0	4.0
F/1a	7	4 ± 1§	3 ± 1	3 ± 1	4 ± 1	3 ± 1
	14	6 ± 1	6 ± 2	5 ± 1	6 ± 1	5 ± 1
	21	10 ± 2	9 ± 2	10 ± 2	9 ± 2	8 ± 1
F/1b	7	4 ± 1	4 ± 1	4 ± 1	4 ± 1	4 ± 1
	14	6 ± 1	7 ± 2	6 ± 1	7 ± 2	7 ± 1
	21	9 ± 3	10 ± 3	9 ± 2	9 ± 2	9 ± 2
F/1c	7	¶	4 ± 1	5 ± 1	5 ± 1	4 ± 1
	14	¶	6 ± 2	8 ± 2	7 ± 1	6 ± 1
	21	¶	10 ± 4	11 ± 3	9 ± 3	9 ± 1
F/2a	7	3 ± 1	4 ± 1	3 ± 1	3 ± 1	2#
	14	5 ± 1	6 ± 2	5 ± 1	5 ± 1	4#
	21	7 ± 2	8 ± 2	7 ± 2	6 ± 2	5#
F/2b	7	¶	4 ± 1	4 ± 1	4 ± 1**	3 ± 1**
	14	¶	7 ± 2	6 ± 1**	5 ± 1**	5 ± 1**
	21	¶	9 ± 2	7 ± 2**	6 ± 1**	6 ± 1**

* Naive control

† Vehicle control

§ Mean of litter means ± SD (g)

¶ Animals used for teratology and dominant lethal studies

** Significantly different from vehicle control, Dunnett's T-test, $p \leqq 0.05$.

Taken from Riddle _et_ _al_., in press.

TCM (Table 16) also decreased the body weights in the F/2a generation in a treatment-related manner. With the exception of the 5.0 mg/ml, F/1b group, TCM was without effects on body weight gain in the other generations. With the exception of the F/2b generations, a significant reduction in pup postnatal survival (LI) was not correlated with a significant decrease in pup body weight gain.

Necropsy --

Gross necropsy was performed on the 21-day old pups that did not serve as parents for the next generation. One male and one female were randomly selected from each litter for gross necropsy. No abnormalities were noted in these animals.

Dominant Lethal Screening --

DBCM and TCM produced no statistically significant dominant lethal effects in the F/1c and F/2b offspring of male mice ingesting these chemicals (Tables 17 and 18, respectively). There were no significant effects on the number of early resorptions, late resorptions or total resorptions (data not shown). The control values for the percentage of implants resorbed were higher than the control values obtained in previous studies. An explanation for these observations was not evident. The limited number of litters prevented a rigorous statistical evaluation of the data. (In the F/1c and F/2b generations, the litters were divided between the dominant lethal, teratology and 21-day survival studies.)

Teratology Screening --

DBCM and TCM produced no statistically significant teratologic effects in the F/1c and F/2b offspring of female mice ingesting these chemicals (Tables 19 and 20, respectively). The number of total implantations and live fetuses/litter appeared to be decreased by the high concentrations of both DBCM and TCM in both generations. However, this was not statistically significant.

The frequency distribution of gross, visceral, or skeletal malformations in the offspring of mice ingesting DBCM or TCM (Tables 21 and 22) was not statistically significant. Gross variations observed included spina bifida, absence of calvarium, and talipes. Skeletal variations observed included delayed ossification, rudimentary ribs, displastic bones, and foreshortened snout. Soft tissue variations included cardiac hypertrophy, bladder hypertrophy, hydronephrosis, hydroureter, and dilated renal pelvis. These variations were equally distributed between the control and treatment groups of both generations.

TCM and DBCM represent major contaminants in municipal drinking water supplies. In an attempt to assess the risks to the human population, we have evaluated the reproductive, dominant lethal and teratologic effects of these materials in mice. The route of exposure (i.e., in the drinking water) was chosen to simulate one route of human exposure. Dosage levels were chosen (based on acute and 90-day studies) which greatly exceeded the

TABLE 16
POSTNATAL BODY WEIGHT AMONG OFFSPRING OF MICE INGESTING TCM

		Concentration (mg/ml)				
Generation	Day	0.0*	0.0†	0.1	1.0	5.0
F/1a	7	4 ± 1§	3 ± 1	4 ± 1	5 ± 1	4 ± 1
	14	6 ± 1	6 ± 2	5 ± 1	7 ± 1	6 ± 1
	21	10 ± 2	9 ± 2	10 ± 2	10 ± 2	8 ± 1
F/1b	7	4 ± 1	4 ± 1	5 ± 1	5 ± 1	4 ± 1
	14	6 ± 1	7 ± 2	7 ± 1	7 ± 2	6 ± 1
	21	9 ± 2	10 ± 3	11 ± 2	11 ± 3	8 ± 2¶
F/1c	7	#	4 ± 1	5 ± 1	4 ± 1	4 ± 1
	14	#	7 ± 1	7 ± 1	6 ± 1	6 ± 1
	21	#	9 ± 2	10 ± 2	9 ± 2	8 ± 2
F/2a	7	4 ± 1	4 ± 1	4 ± 1	4 ± 1	4 ± 1
	14	5 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1
	21	7 ± 2	8 ± 2	7 ± 1	7 ± 1	7 ± 1
F/2b	7	#	4 ± 1	4 ± 1	4 ± 1¶	3 ± 1¶
	14	#	7 ± 2	6 ± 1¶	5 ± 1¶	6 ± 1¶
	21	#	9 ± 2	8 ± 2	6 ± 1¶	7 ± 2¶

* Naive control

† Vehicle control

§ Mean of litter means ± SD (g)

¶ Significantly different from vehicle control, Dunnett's T-test, $p \leqq 0.05$.

# Animals used for teratology and dominant lethal studies

Taken from Riddle _et al._, in press.

TABLE 17. RESULTS OF DOMINANT LETHAL SCREENING IN MICE INGESTING DBCM

	F/1c Generation					F/2b Generation				
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	4.0	0.0*	0.0†	0.1	1.0	4.0
Number of Litters	6	5	5	4	4	3	6	6	6	4
Implantations/ Dam§	15 ± 3	12 ± 6	16 ± 2	15 ± 3	16 ± 3	9 ± 6	10 ± 5	11 ± 6	14 ± 2	7 ± 7
Live Fetuses/ Litter§	13 ± 6	10 ± 6	15 ± 2	12 ± 4	13 ± 7	4 ± 7	8 ± 6	11 ± 5	13 ± 2	5 ± 7
% of Implants Resorbed¶	17	29	6	21	23	61	23	4	8	36
Fetal Body (Weight (g)§	1.33 ±0.39	1.13 ±0.43	1.20 ±0.35	1.08 ±0.16	1.23 ±0.24	1.76 ±0.38	0.98 ±0.37	1.30 ±0.40	1.42 ±0.09	0.88 ±0.18

* Naive control

† Vehicle control

§ Mean ± SD

¶ Percentage of Implants Resorbed = $\frac{\text{\# Resorptions}}{\text{\# Implants}} \times 100$

Taken from: Riddle *et al.*, in press.

TABLE 18. RESULTS OF DOMINANT LETHAL SCREENING IN MICE INGESTING TCM

	F/1c Generation					F/2b Generation				
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	5.0	0.0*	0.0†	0.1	1.0	5.0
Number of Litters	6	5	4	4	4	3	6	6	6	3
Implantations/ Dam§	15 ± 3	12 ± 6	16 ± 2	6 ± 9	10 ± 6	9 ± 6	10 ± 5	13 ± 2	13 ± 3	13 ± 3
Live Fetuses/ Litter§	13 ± 6	10 ± 6	15 ± 3	5 ± 8	10 ± 6	4 ± 7	8 ± 6	8 ± 7	11 ± 3	2 ± 6
% of Implants Resorbed¶	17	29	7	12	3	61	23	35	13	84
Fetal Body Weight (g)§	1.33 ±0.39	1.13 ±0.48	1.09 ±0.50	1.33 ±0.28	0.77 ±0.26	1.76 ±0.38	0.93 ±0.37	1.39 ±0.19	1.22 ±0.38	1.20 ±0.06

* Naive control

† Vehicle control

§ Mean ± SD

¶ Percentage of Implants Resorbed = $\frac{\text{\# Resorptions}}{\text{\# Implants}}$ x 100

Taken from: Riddle *et al.*, in press.

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TABLE 19. RESULTS OF TERATOLOGY SCREENING IN MICE INGESTING DBCM

	F/1c Generation					F/2b Generation				
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	4.0	0.0*	0.0†	0.1	1.0	4.0
Number of Litters	6	6	6	6	6	3	6	6	6	6
Implantations/ Dam§	13 ± 2	16 ± 5	13 ± 6	11 ± 6	8 ± 6	10 ± 6	11 ± 6	12 ± 2	12 ± 2	7 ± 6
Live Fetuses/ Litter§	13 ± 2	11 ± 3	12 ± 3	10 ± 3	7 ± 3	8 ± 4	11 ± 3	10 ± 3	4 ± 3	5 ± 3
% of Implants Resorbed¶	7	31	7	9	13	20	3	16	66	29
Sex Ratio M:F	46:54	38:62	50:50	50:50	40:60	50:50	47:53	52:48	50:50	50:50

* Naive control

† Vehicle control

§ Mean ± SD

¶ Percentage of Implants Resorbed = $\frac{\text{\# Resorptions}}{\text{\# Implants}}$ x 100

Taken from: Riddle *et al.*, in press.

TABLE 20. RESULTS OF TERATOLOGY SCREENING IN MICE INGESTING TCM

	F/1c Generation					F/2b Generation				
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	5.0	0.0*	0.0†	0.1	1.0	5.0
Number of Litters	6	6	6	6	6	3	6	6	6	6
Implantations/ Dam§	13 ± 2	16 ± 5	12 ± 6	12 ± 2	13 ± 1	10 ± 6	11 ± 6	13 ± 2	14 ± 4	12 ± 3
Live Fetuses/ Litter§	13 ± 2	11 ± 3	8 ± 4	8 ± 3	7 ± 3	8 ± 4	11 ± 3	9 ± 4	11 ± 4	7 ± 3
% of Implants Resorbed¶	7	31	33	33	38	20	3	31	21	42
Sex Ratio M:F	46:54	38:62	40:60	50:50	50:50	50:50	47:53	47:53	51:49	50:50

* Naive control

† Vehicle control

§ Mean ± SD

¶ Percentage of Implants Resorbed = $\frac{\text{\# Resorptions}}{\text{\# Implants}}$ x 100

Taken from: Riddle et al., in press.

TABLE 21. INCIDENCE OF FETAL DEVELOPMENTAL VARIATIONS IN THE F/1c LITTERS OF MICE INGESTING DBCM AND TCM

GROUP	CONTROL		DBCM			TCM		
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	4.0	0.1	1.0	5.0
No. Fetuses Examined (No. Litters)	75 (6)	67 (6)	70 (6)	49 (6)	44 (6)	50 (6)	46 (6)	60 (6)
No. Gross Malformations (No. Litters)	1 (1)	0	0	1 (1)	1 (1)	0	1 (1)	1 (1)
No. Visceral Variations (No. Litters)	1 (1)	0	0	1 (1)	1 (1)	1 (1)	2 (2)	1 (1)
No. Skeletal Variations (No. Litters)	0	4 (1)	0	4 (2)	0	7 (2)	3 (1)	4 (1)
Total Variations (No. Litters)	1 (1)	4 (1)	0	6 (4)	2 (2)	8 (3)	6 (4)	6 (3)

* Naive Control

† Vehicle = Emulphor:water (1:1000)

Taken from: Riddle _et_ _al_., in press.

TABLE 22. INCIDENCE OF FETAL DEVELOPMENTAL VARIATIONS IN THE F/2b LITTERS OF MICE INGESTING DBCM AND TCM

GROUP	CONTROL		DBCM			TCM		
Concentration (mg/ml)	0.0*	0.0†	0.1	1.0	4.0	0.1	1.0	5.0
No. Fetuses Examined (no. litters)	24 (3)	65 (6)	59 (6)	25 (6)	32 (6)	53 (6)	68 (6)	20 (6)
No. Gross Malformations	0	0	0	0	1	0	0	0
No. Viseral Variations (no. litters)	0	0	1 (1)	0	0	0	0	0
Total Variations (no. litters)	0	0	1 (1)	0	1 (1)	1 (1)	0	0

* Naive Control

† Vehicle = Emulphor:water (1:1000)

Taken from: Riddle *et al.*, in press.

normal daily human consumption. Ingestion of these materials at concentrations of 4.0 mg/ml (DBCM) and 5.0 mg/ml (TCM) produced significant decreases in body weight gain and survival in males and females of the F/0 and F/1b generations. Upon necropsy, these animals exhibited a liver pathology characteristic of chlorinated hydrocarbon toxicity.

A summary of the results of this study is presented in Table 23. Significant maternal toxicity was observed in the F/0 and F/1b generations exposed to the high concentrations of both test materials. The existence of embryotoxicity was indicated by the effects on fertility, litter size, and the gestation index. Postnatal survival was marginally affected by the high concentrations of both test materials (the viability index more so than the lactation index). Although no statistically significant effects were observed in the teratology studies, the number of total implants and live fetuses per litter appeared to be decreased by the high concentrations of both test materials. These trends suggested that the effects observed on reproduction may have been due to a toxic effect on the dams. That is, the embryotoxicity was due to the maternal toxicity. The high percentage of implants resorbed in the treated groups may have been responsible for the decrease in the fertility index. The decrease in the average number of pups per litter at birth might also have been explained in terms of an increase in the incidence of resorptions. The effects on viability and lactation might also have been due to maternal toxicity and a concurrent decreased development.

The 1 mg/ml concentrations of DBCM and TCM produced only marginal toxicity in the F/0 and F/1b generations. The females appeared more sensitive to the toxic effects than the males. The reproductive parameters appeared only sporadically affected. The 0.1 mg/ml concentrations of both compounds were without effect on any of the reproductive, teratologic or dominant lethal parameters, with the exception of postnatal body weight in the F/2b generation. In the F/2a and F/2b generations, it appears that there was a concentration-dependent decrease in weight gain. It was not clear if this was due to an initial maternal-embryotoxicity at birth or a postnatal toxicity associated with ingestion of the test materials.

There do not appear to be any multigenerational effects associated with the ingestion of either DBCM or TCM. The effects observed in offspring generally were also seen in the parents. The severity of the effects in the offspring was the same as in the parents. In most cases, effects decreased in severity from the first generation to the second generation.

The lack of teratogenicity of TCM in this study is consistent with previous studies. Schwetz _et al_. (1974) investigated the teratology of inhaled TCM in gestating rats. At concentrations ranging from 30 to 300 ppm, TCM was found to be highly embryotoxic when exposures were 7 hr/day during gestation Days 6-15. Exposure to 300 ppm resulted in some maternal toxicity and substantial fetotoxicity. The authors concluded that TCM was not highly teratogenic. Thompson _et al_. (1974) studied the teratogenicity of TCM in rats and rabbits. They found no major external, skeletal, or viseral malformations when TCM was given orally in doses ranging from

TABLE 23. SUMMARY OF EFFECTS OF DBCM AND TCM

Parameter	Generation	DBCM*			TCM*		
		0.1	1.0	4.0	0.1	1.0	5.0
Body Weight Gain							
(Males)	F/0	0†	0	↓§	0	0	↓
	F/1	0	0	↓	0	0	↓
(Females)	F/0	0	↓	↓	0	0	↓
	F/1	0	↓	↓	0	↓	↓
Survival							
(Males)	F/0	0	0	0	0	0	↓
	F/1	0	0	↓	0	↓	↓
(Females)	F/0	0	0	0	0	0	↓
	F/1	0	0	↓	0	0	↓
Fertility	F/0 → F/1a	0	0	0	0	0	↓
	F/0 → F/1b	0	0	0	0	0	↓
	F/0 → F/1c	0	0	0	0	0	↓
	F/1b → F/2a	0	0	↓	0	0	↓
	F/1b → F/2b	0	0	0	0	↓	↓
Litter Size	F/1a	0	0	↓	0	0	↓
	F/1b	0	0	↓	0	0	↓
	F/1c	0	↓	↓	0	0	↓
	F/2a	0	0	↓	0	0	↓
	F/2b	0	0	↓	0	0	↓
Gestation Index	F/0 → F/1a	0	0	↓	0	0	↓
	F/0 → F/1b	0	0	↓	0	0	0
	F/0 → F/1c	0	0	↓	0	0	↓
	F/1b → F/2a	0	0	0	0	0	↓
	F/1b → F/2b	0	0	0	0	0	0
Viability Index	F/1a	0	0	↓	0	0	↓
	F/1b	0	↓	↓	0	↓	↓
	F/1c	0	0	↓	0	0	0
	F/2a	0	0	↓	0	0	↓
	F/2b	0	0	0	0	0	0

* Concentration (mg/ml)
† 0 = No change
§ ↓ = Decrease from control value

(Continued)

TABLE 23. (Continued)

Parameter	Generation	DBCM*			TCM*		
		0.1	1.0	4.0	0.1	1.0	5.0
Lactation Index	F/1a	0†	0	0	0	↓§	↓
	F/1b	0	↓	0	0	0	0
	F/1c	0	0	0	0	0	0
	F/2a	0	0	0	0	0	0
	F/2b	0	↓	↓	0	0	↓
Postnatal Body Weight	F/1a	0	0	0	0	0	0
	F/1b	0	0	0	0	0	↓
	F/1c	0	0	0	0	0	0
	F/2a	0	0	0	0	0	0
	F/2b	↓	↓	↓	0	↓	↓
Dominant Lethal	F/1c	0	0	0	0	0	0
	F/2b	0	0	0	0	0	0
Teratology	F/1c	0	0	0	0	0	0
	F/2b	0	0	0	0	0	0

* Concentration (mg/ml)
† 0= No Change
§ ↓= Decrease from control value

Taken from: Riddle *et al*., in press.

20-50 mg/kg/ day during gestation Days 6-15 in the rat. Burkhalter and Balster (1979) evaluated the possible behavioral effects associated with mice treated (gavage) with TCM throughout reproduction (prior to and throughout mating, gestation and lactation). The pups were evaluated for neurobehavioral development, motor performance, and learning. No consistent differences were observed in any of the measures. Dilley *et al.* (1977) reported that rats exposed to TCM (20 g/m^3; days 7-14 of gestation) by inhalation did not demonstrate teratogenic responses. Significant fetal mortality and decreased fetal weight gain were observed.

The results of this study are not in agreement with the data of Murray *et al.* (1979). This group exposed CF-1 mice to 0 or 100 ppm for 7 hr/day from days 1 through 7, 6 through 15, or 8 through 15 of gestation. Exposure to TCM from days 1 through 7 or 6 through 15 significantly impaired the ability of the female mice to maintain pregnancy but was not significantly teratogenic. In comparison, a significant increase in the incidence of cleft palate was observed in mice inhaling TCM from Days 8 through 15 of gestation, but no effect on the ability of the dam to maintain pregnancy was discerned. The finding of fetal anomalies following exposure of pregnant mice to TCM by inhalation and their absence in our studies following administration in the drinking water may be attributed to a number of differences in the two studies. Blood concentrations and tissue distribution of TCM in maternal and fetal compartments would undoubtedly be affected by the strain of mouse, route and duration of maternal exposure. The parameters differed in the two studies, i.e., continuous exposure of CF-1 mice 7 hr/day during gestation via inhalation as compared to long term (approximately 24 weeks) ingestion by ICR mice. A measurement of the blood levels and degree of metabolism under the two experimental conditions would offer a meaningful point of comparison.

Although reproductive toxicity was observed in the present studies on DBCM and TCM, the concentrations tested were considerably greater than those which might be ingested by humans through drinking contaminated water. The maximum observed levels in finished drinking water supplies of DBCM and TCM were 116 µg/l and 311 µg/l, respectively. If one assumes that the average human weighs 70 kg and consumes 2 l of water per day, the consumption on a per kg of body weight per day basis are calculated as 3.31 µg/kg/ day (DBCM) and 8.89 µg/kg/day (TCM). Assuming that the average mouse weighs 30 g and consumes 5 ml of water per day, the corresponding consumption of the test drinking water solutions are calculated to be 20,000, 200,000, 800,000 (DBCM) and 20,000, 200,000, 1,000,000 (TCM) µg/kg/day. Thus, the mice were exposed to 5,000, 50,000 or 240,000 (DBCM) times the normal human exposure levels. (The associated values for TCM are 2,200, 22,000 and 110,000.) Only the high concentrations of DBCM and TCM consistently affected reproduction. No effects were observed at the lower concentrations. Thus, the presence of DBCM and TCM in drinking water at commonly occurring levels is not likely to present a reproductive hazard. If any reproductive hazard to humans does exist, it is most likely to be in the case of an overexposure such as an intentional or accidental poisoning with the chemical at high concentrations.

Taken From: Riddle *et al.*, in press.

INHIBITION OF TESTICULAR DNA SYNTHESIS

Positive-Controls

Methyl-methanesulfonate (MMS) --

A positive control was included in all experiments. In the first series of experiments, methyl-methanesulfonate was used. It was administered via three different routes, oral gavage (o.g.), intraperitoneal injection, (i.p.). and intratesticular injection (i.t.) A comparison of the relative efficacies of these routes was made, and these data are presented in Tables 24 and 25 and graphed in Figure 18. The three upper lines trace the effect of MMS upon tritiated thymidine pool size. Only at the 100mg/kg dose, administered intratesticularly, is a significant deviation from the vehicle control values observed. The three lower lines indicate the effects upon testicular DNA synthesis. This effect is dose-related, and also route-related. The order of decreasing induced effect upon testicular DNA synthesis via the three routes tested is: intratesticular injection, intraperitoneal injection, and oral gavage. These data indicate that the intratesticular (i.t.) route of administration gives a truer indication of maximal effect possible from a given dose. For this reason, the i.t. route of administration was used for all subsequent experiments.

Figure 19 (data, Tables 26 and 27) once again depicts the effects of MMS upon testicular DNA synthesis. Each of the points on this curve was obtained from an averaging of the data from all experiments performed at the given dose. Because these points fall on the same curve, this figure also demonstrates the reproducibility of the inhibition of DNA synthesis assay. As has been previously demonstrated, i.t. administration of MMS produced a dose-related increase in tritiated thymidine pool size and a decrease in testicular DNA synthesis.

Daunorubicin (DNR) --

In later experiments, daunorubicin was used. X-ray diffraction and other analyses have suggested that daunorubicin may intercalate between adjacent base pairs of the DNA helix, causing an untwisting of the DNA helix and rendering it inactive as a template for DNA replication (Gilman *et al.*, 1980). With this in mind, a dose-related decrease in testicular DNA synthesis and concommitant increase in tritiated thymidine pool size were expected.

The hypothesis that daunorubicin would cause a decrease in testicular DNA synthesis was supported by the data. Doses as low as 0.025 mg/kg, administered i.t., caused a significant deviation from the vehicle control values (Dunnett's and Duncan's tests). Doses of 1.0 mg/kg and greater caused a significant increase in tritiated thymidine pool size. As each of these data points is from a different experiment, the graph in Figure 20 once again confirms the reproducibility of the DNA synthesis assay.

Experimentals

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TABLE 24. EFFECTS OF DIFFERENT ROUTES OF ADMINISTRATION OF MMS UPON MOUSE TESTICULAR DNA SYNTHESIS

ROUTE ADMIN	DOSE (mg/kg)	DPMS1 Mean	DPMS1 % ± SEM^{N}	DPMSF Mean	DPMSF % ± SEM^{N}	DNAMICG Mean	DNAMICG % ± SEM^{N}
i.t.	0	7428759	100 ± 12^{4}	3103020	100 ± 10^{4}	5392	100 ± 6^{4}
	25	7597567	102 ± 12^{4}	615243	10 ± 3^{4}	5854	109 ± 5^{4}
	50	6448711	87 ± 11^{4}	139872	5 ± 1^{4}	5091	94 ± 8^{4}
	100	12476730	168 ± 4^{4}	78303	3 ± 0^{4}	6425	119 ± 6^{4}
o.g.	0	7966324	100 ± 3^{4}	2799285	100 ± 8^{4}	5616	100 ± 13^{4}
	25	8272334	104 ± 4^{4}	2809356	100 ± 16^{4}	6604	118 ± 2^{4}
	50	9451730	119 ± 9^{4}	2347625	84 ± 22^{4}	6388	114 ± 18^{4}
	100	8336311	105 ± 12^{4}	1859187	66 ± 7^{4}	6139	109 ± 19^{4}
i.p.	0	8945118	100 ± 8^{4}	3649421	100 ± 8^{4}	5950	100 ± 8^{4}
	25	12190844	136 ± 18^{4}	2216340	61 ± 11^{4}	6320	106 ± 2^{4}
	50	8025164	90 ± 10^{4}	1090816	30 ± 6^{4}	5115	86 ± 5^{4}
	100	7771063	87 ± 16^{4}	934351	26 ± 8^{4}	5569	94 ± 13^{4}

TABLE 25. STATISTICAL ANALYSIS OF THE EFFECTS OF DIFFERENT ROUTES OF ADMINISTRATION OF MMS UPON MOUSE TESTICULAR DNA SYNTHESIS

ROUTE ADMIN.	DOSE (mg/kg)	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
		LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
i.t.	0	.09		.22	A	.01		.01	B	.00		.00	A	.15		.10	AB	.12		.09	AB
	25				A				B		+		B				AB				AB
	50				A				B		+		C				B				B
	100				A		+		A		+		C				A				A
o.g.	0	.17		.39	A	.75		.45	A	.05		.28	A	.92		.83	A	.79		.86	A
	25				A				A				A				A				A
	50				A				A				A				A				A
	100				A				A				A				A				A
i.p.	0	.65		.36	A	.14		.09	AB	.00		.00	A	.27		.29	A	.37		.36	A
	25				A				A				A				A				A
	50				A				B		+		B				A				A
	100				A				B		+		B				A				A

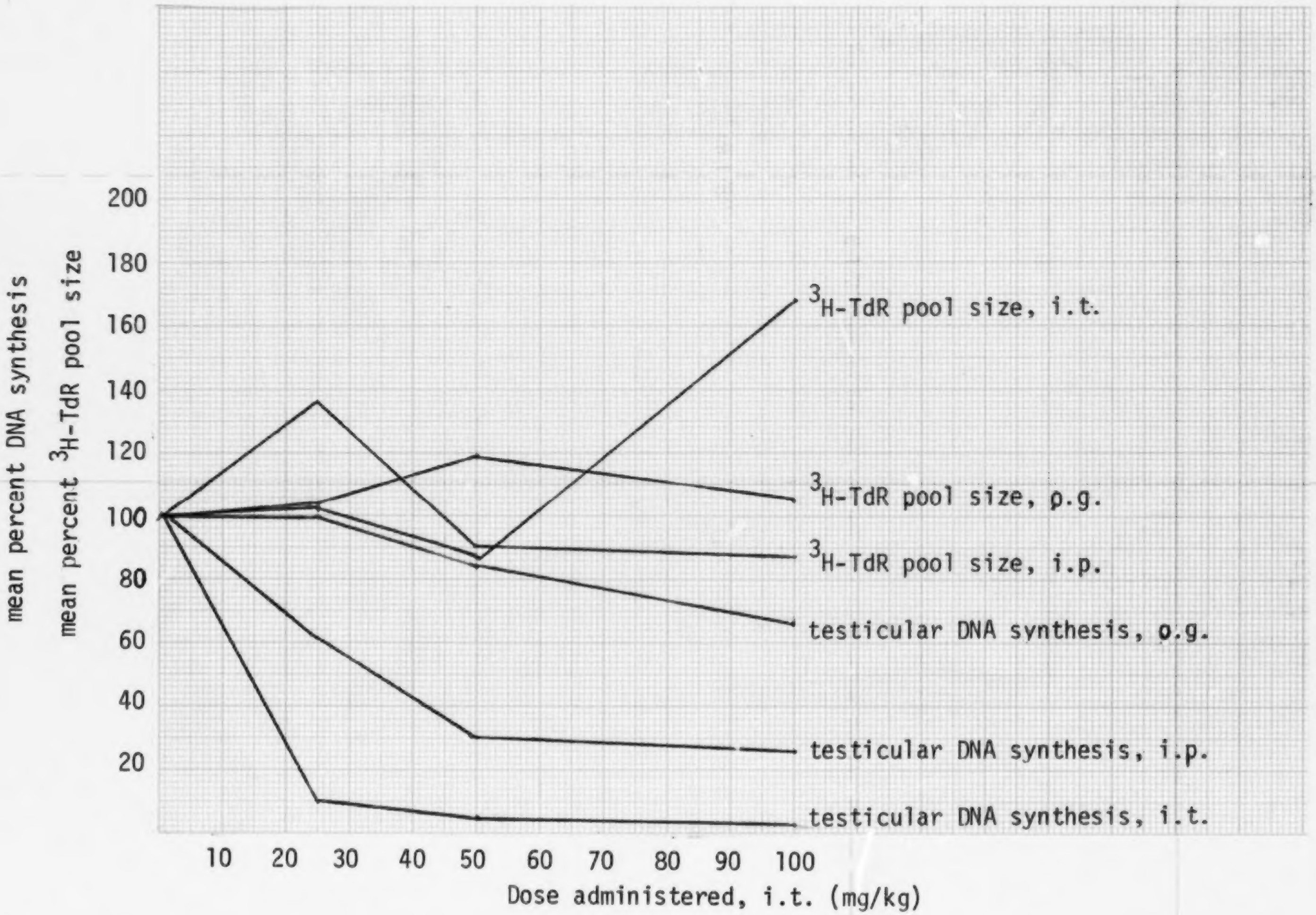

Figure 18. Effects of different routes of administration of MMS upon mouse testicular DNA synthesis.

TABLE 26. EFFECTS OF MMS UPON MOUSE TESTICULAR DNA SYNTHESIS

DOSE (mg/kg)	DPMS1		DPMSF		DNAMICG	
	Mean	% ± SEM^{N}	Mean	% ± SEM^{N}	MEAN	% ± SEM^{N}
0	11885558	100 ± 5^{4}	4521408	100 ± 19^{4}	5996	100 ± 10^{4}
5	14744877	124 ± 5^{4}	2354471	52 ± 15^{4}	4644	77 ± 10^{4}
0	9932055	100 ± 7^{7}	2391525	100 ± 8^{7}	4587	100 ± 6^{7}
2.5	10940606	110 ± 14^{7}	1749814	73 ± 13^{7}	4459	97 ± 14^{7}
0	14832369	100 ± 10^{7}	4949105	100 ± 4^{7}	5660	100 ± 5^{7}
10	16971668	114 ± 6^{7}	2476368	50 ± 7^{7}	5616	99 ± 4^{7}
0	13861880	100 ± 22^{4}	4187899	100 ± 19^{4}	5990	100 ± 4^{4}
10	19264165	139 ± 31^{4}	1903396	45 ± 12^{4}	6170	103 ± 9^{4}
0	7428759	100 ± 12^{4}	3103020	100 ± 10^{4}	5392	100 ± 6^{4}
25	7597567	102 ± 12^{4}	615243	20 ± 3^{4}	5854	109 ± 5^{4}
50	6448711	87 ± 11^{4}	139872	5 ± 1^{4}	5091	94 ± 8^{4}
100	12476730	168 ± 10^{4}	78303	3 ± 0^{4}	6425	119 ± 6^{4}
0	14764602	100 ± 13^{8}	1707707	100 ± 17^{8}	4962	100 ± 6^{8}
10	16801976	114 ± 20^{7}	664424	39 ± 9^{7}	5463	110 ± 11^{7}
50	11285308	76 ± 5^{8}	30783	2 ± 0^{8}	4695	95 ± 5^{8}

TABLE 27. STATISTICAL ANALYSIS OF EFFECTS OF MMS UPON MOUSE TESTICULAR DNA SYNTHESIS

DOSE	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.78		.78	A	.02		.02	B	.13		.13	A	.15		.15	A	.15		.15	A
5				A		+		A				A				A				A
0	.42		.42	A	.73		.73	A	.18		.18	A	.63		.63	A	.86		.86	A
2.5				A				A				A				A				A
0	.10		.10	A	.19		.19	A	.00		.00	A	.94		.94	A	.90		.90	A
10				A				A		+		B				A				A
0	.67		.67	A	.36		.36	A	.07		.07	A	.83		.83	A	.77		.77	A
10				A				A				A				A				A
0	.09		.22	A	.01		.01	B	.00		.00	A	.15		.10	AB	.12		.09	AB
25				A				B		+		B				AB				AB
50				A				B		+		C				B				B
100				A		+		A		+		C				A				A

(Continued)

TABLE 27. (Continued)

Dose	LDPMI				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.01		.03	A	.13		.26	A	.00		.00	A	.39		.44	A	.39		.38	A
10				AB				A		+		B				A				A
50		+		B				A		+		C				A				A

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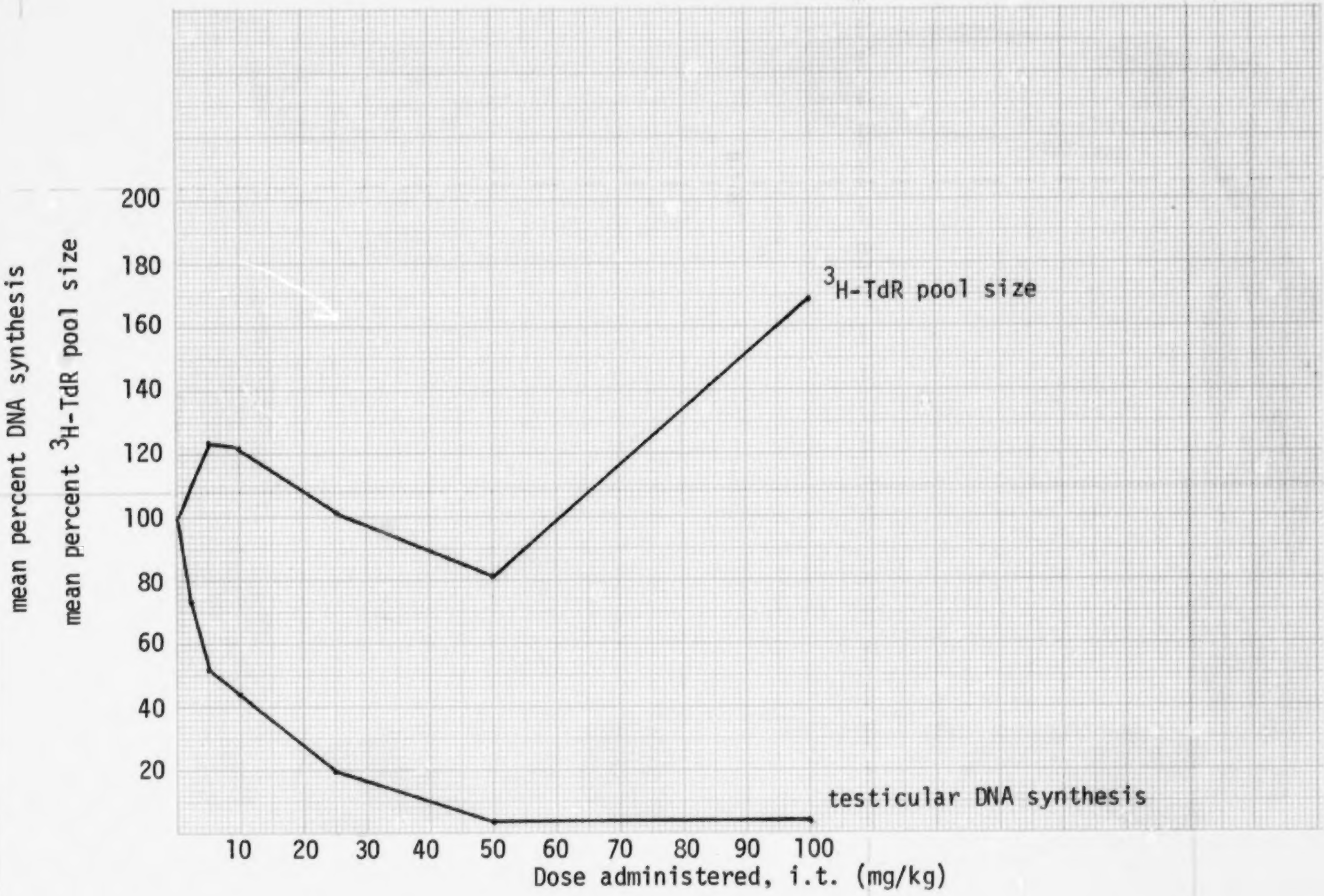

Figure 19. Effects of MMS upon mouse testicular DNA synthesis.

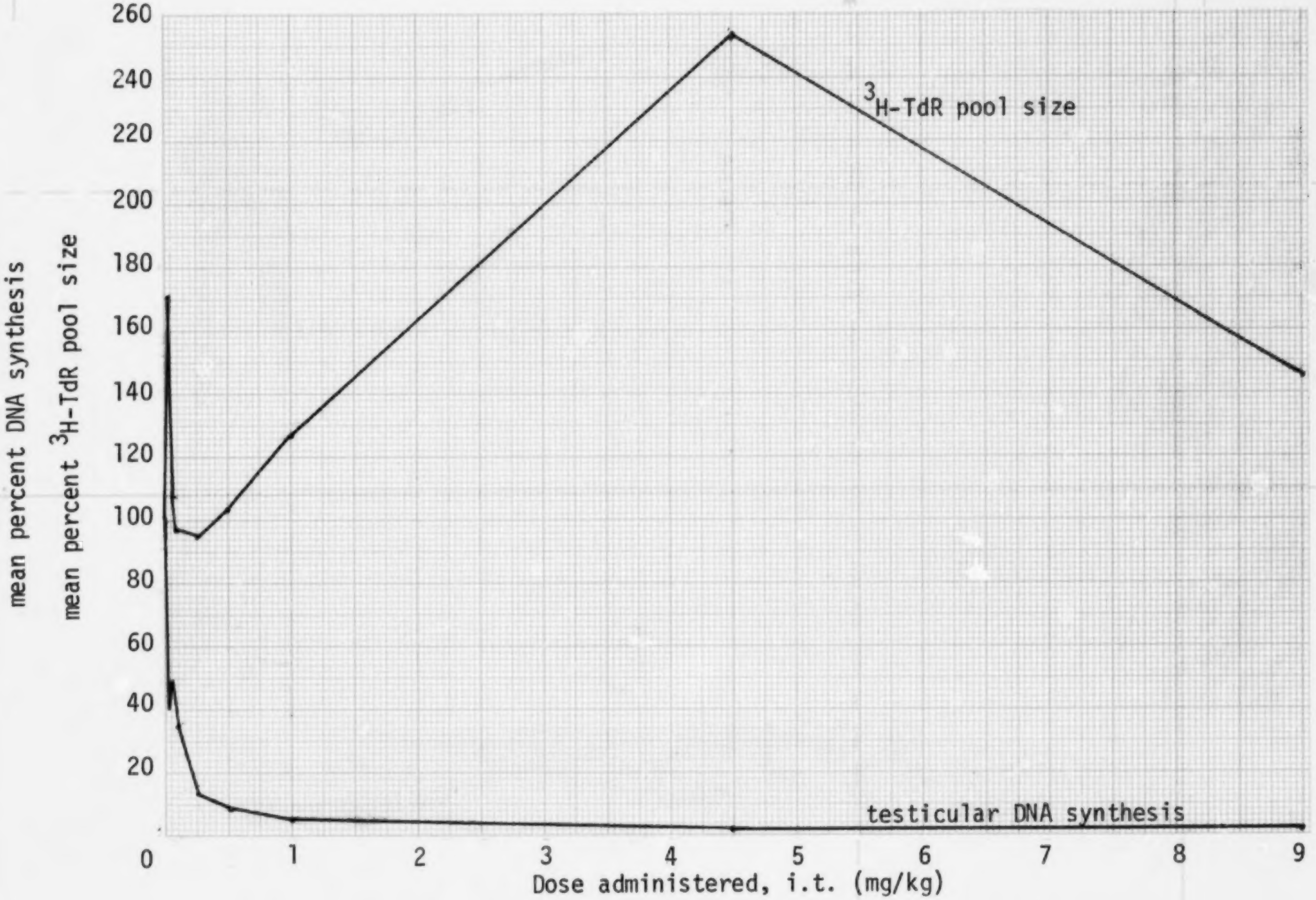

Figure 20. Effects of DNR upon mouse testicular DNA synthesis.

Chloral hydrate (CHL) --

In four separate experiments, chloral hydrate was administered to 12 week old Swiss ICR mice. The data obtained are presented in Table 28, with the statistical analyses of these data presented in Table 29.

The data indicate that the two highest doses tested, 600 and 900 mg/kg, caused a significant increase in mean dpm in the homogenate fraction. The values at the two doses were 158 and 207% of the vehicle control values, respectively. As the counts in the homogenate fraction are a measure of total testicular counts, this data must be interpreted as indicating that high doses of CHL are capable of causing an inhibition of transport of tritiated thymidine out of the testes. This implies the presence of a mechanism, acting through either facilitated diffusion or active transport, which is capable of transporting tritiated thymidine out of the testes.

The data obtained from analyses of the first supernatant fraction show a wide variation in response. Even so, it is apparent that the 600 and 900 mg/kg doses caused a significant increase in tritiated thymidine pool size. The ANOVA p value for this fraction approximates zero and both the Dunnett's and Duncan's tests place the means of the first supernatant fraction of the 600 and 900 mg/kg dose groups apart from those at the other doses tested. The increase in pool size might be expected as a result of the increase in total testicular counts at these doses, accompanied by a decrease in testicular DNA synthesis.

Testicular DNA systhesis is quantitated in the dpm of the final supernatant fraction. In all experiments, doses greater than or equal to 75 mg/kg caused a significant decrease in testicular DNA synthesis. The mean percent of control responses varied from 30% at 75 mg/kg to 3% at 300 mg/kg. The data obtained are graphed in Figure 21.

1,1,2-Trichloroethylene (TCE 2) --

Doses of TCE 2 up to 1000 mg/kg were tested (Tables 30 and 31, Figure 22). No effects upon total testicular counts were elicited. Effects upon tritiated thymidine and testicular DNA synthesis, however, were produced. At doses of 500 mg/kg and above, pool size quantities are greater than control values. Once again, a large variance in the numbers obtained was apparent. As a result, only the pool size of 209% at dose level 750 mg/kg was determined to be statistically significant. Smaller variances in the results were obtained for dpm in the final supernatant fraction. Here, a clearer picture of the effect of the compound upon testicular DNA synthesis is given. The mean percent dpm at the 250, 500, 750, and 1000 mg/kg dose levels were 74, 40, 18, and 2% of the control values, respectively. These last three values were found to deviate significantly from the control values (Dunnett's T-test) and from one another (Duncan's multiple range test).

1,1,2-Trichloroethane (TCE 1,2) --

TABLE 28. EFFECTS OF CHL UPON MOUSE TESTICULAR DNA SYNTHESIS

DOSE	DPMH		DPMS1		DPMSF		DNAMICG	
(mg/kg)	Mean	% ± SEM^{N}	Mean	% ± SEM^{N}	Mean	% ± SEM^{N}	Mean	% ± SEM^{N}
0	24524762	100 ± 19^{6}	24492603	100 ± 21^{6}	2911257	100 ± 24^{6}	3592	100± 13^{6}
350	12052089	49 ± 11^{6}	11731223	48 ± 11^{6}	40019	1 ± 0^{6}	2756	77± 7^{6}
700	18381253	75 ± 8^{6}	18633300	76 ± 8^{6}	18524	1 ± 0^{6}	3881	108± 37^{6}
0	16484957	100 ± 10^{7}	14832369	100 ± 10^{7}	4949105	100± 4^{7}	5660	100± 5^{7}
75	13297132	81 ± 10^{7}	12337035	83 ± 11^{7}	980912	20± 5^{7}	5686	100± 6^{7}
150	15202690	92 ± 9^{7}	13349593	90 ± 9^{7}	378480	8± 2^{7}	6084	107± 5^{7}
300	12905982	78 ± 8^{7}	11452359	77 ± 8^{7}	145120	3± 1^{7}	5164	91± 8^{7}
600	25967823	158 ± 16^{7}	23261618	157 ± 18^{7}	113846	2± 1^{6}	5519	98± 1^{7}
900	34047026	207 ± 21^{6}	31606383	213 ± 21^{6}	35585	1± 0^{6}	5545	98± 6^{6}
0	14448927	100 ± 7^{6}	14787583	100 ± 6^{7}	5877007	100± 7^{7}	5655	100± 4^{7}
10	17007881	118 ±30^{7}	18071384	122 ± 31^{7}	5899865	100± 9^{7}	5475	97± 4^{7}
25	13266218	92 ±10^{6}	13925568	94 ± 4^{6}	5361303	91± 10^{6}	5624	99± 8^{6}
50	8011751	55 ±6^{7}	7768244	53 ± 5^{7}	3098943	53± 4^{7}	5519	98± 6^{7}
75	14951164	103 ±15^{7}	15517855	105 ± 15^{7}	2183147	37± 5^{7}	6154	109± 5^{7}
150	19047618	132 ±22^{7}	18817013	127 ± 22^{7}	475319	8± 1^{6}	5822	103± 3^{6}
0			21352366	100 ± 27^{5}	1748006	100± 32^{5}	5039	100± 8^{5}
56			8067065	38 ± 2^{6}	776482	44± 5^{6}	4537	90± 4^{6}
474			18955415	89 ± 17^{6}	30906	2± 0^{6}	4729	94± 4^{6}

TABLE 29. STATISTICAL ANALYSIS OF EFFECTS OF CHL UPON MOUSE TESTICULAR DNA SYNTHESIS

DOSE	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.46		.03	A	.53		.03	A	.00		.00	A	.81		.64	A	.81		.62	A
350		+		B		+		B		+		B				A				A
700				AB				AB		+		C				A				A
0	.00		.00	B	.00		.00	B	.00		.00	A	.54		.40	A	.49		.51	A
75				B				B		+		B				A				A
150				B				B		+		B				A				A
300				B				B		+		C				A				A
600		+		A		+		A		+		CD				A				A
900		+		A		+		A		+		D				A				A
0	.31		.01	A	.50		.00	A	.00		.00	A	.28		.57	A	.30		.58	A
10				A				A				A				A				A
25				A				A				A				A				A
50		+		B		+		B		+		B				A				A
75				A				A		+		C				A				A
150				A				A		+		D				A				A
0	.49		.08	A	.38		.02	A	.00		.00	A	.86		.50	A	.81		.46	A
56				A		+		B				A				A				A

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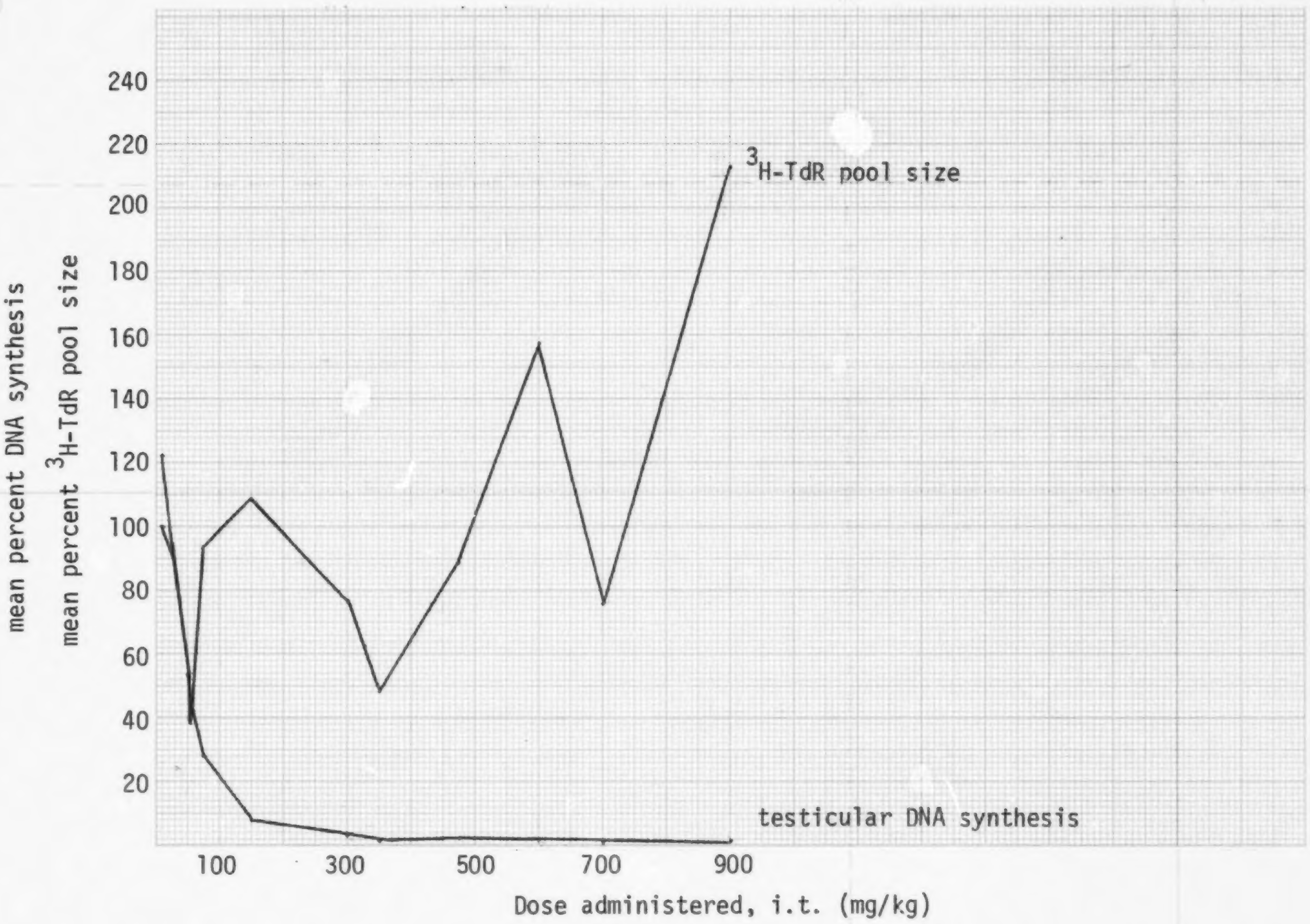

Figure 21. Effects of CHL upon mouse testicular DNA synthesis.

TABLE 30. EFFECTS OF TCE 2 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	DPMH		DPMS1		DPMSF		DNAMICG	
(mg/kg)	Mean	% ± SEM^N	Mean	% ± SEM^N	Mean	% ± SEM^N	Mean	% ± SEM^N
0			16384966	100 ± 18^6	3630460	100 ± 13^6	5756	100 ± 8^6
24			19288795	118 ± 19^5	3575362	98 ± 11^5	6348	110 ± 4^5
240			15902881	97 ± 24^5	3581628	99 ± 8^5	5821	101 ± 9^5
0			10272538	100 ± 25^7	2260051	100 ± 17^7	4246	100 ± 15^7
250			8205036	80 ± 20^7	1668723	74 ± 14^7	4830	114 ± 8^7
500			13609083	132 ± 23^7	894706	49 ± 7^6	4871	115 ± 8^7
750			21481683	209 ± 21^7	402328	18 ± 3^6	4615	109 ± 6^7
1000			17927535	175 ± 22^6	44885	2 ± 0^6	4831	114 ± 9^6
0	14425580	100 ± 20^5	12761323	100 ± 13^5	2846876	100 ± 17^5	5013	100 ± 6^5
24	12066667	84 ± 13^5	13141622	103 ± 10^5	3106282	109 ± 17^5	4832	96 ± 8^5
240	8474562	59 ± 7^5	8900323	70 ± 9^5	1640409	58 ± 17^5	4624	92 ± 16^5

TABLE 31. STATISTICAL ANALYSIS ON EFFECTS OF TCE 2 UPON TESTICULAR DNA SYNTHESIS

Dose	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.24		.13	A	.54		.62	A	.92		.99	A	.76		.51	A	.77		.58	A
24				A				A				A				A				A
240				A				A				A				A				A
0	.14		.23	AB	.00		.00	B	.00		.00	A	.26		.57	A	.46		.80	A
250				B				B				A				A				A
500				AB				AB		+		B				A				A
750				A		+		A		+		C				A				A
1000				AB				A		+		D				A				A
0	.05		.14	A	.04		.13	A	.02		.08	AB	.42		.72	A	.64		.89	A
24				A				A				A				A				A
240				A				A				A				A				A

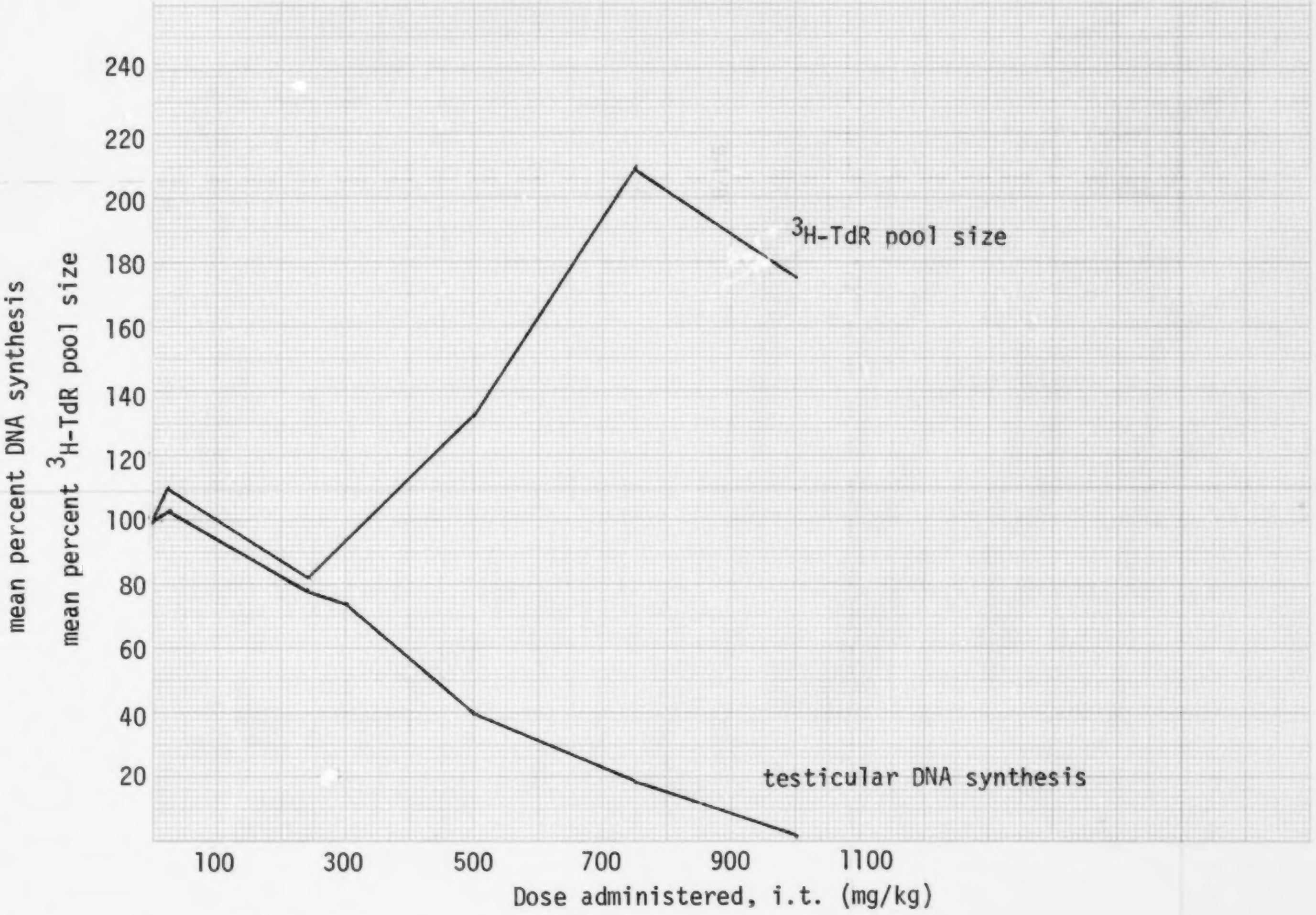

Figure 22. Effects of TCE 2 upon mouse testicular DNA synthesis.

Concentrations of TCE 1,2 ranging from 3.8 to 1200 mg/kg were tested for their effects upon murine testicular DNA synthesis. In general, a dose-related increase in tritiated thymidine pool size and decrease in testicular DNA synthesis is observed. At a dose of 300 mg/kg, tritiated thymidine pool size is 1.7 times that of the control value, while DNA synthesis is 1/3 that of the control. In general, these effects become statistically significant at doses greater than or equal to 300 mg/kg. These data are presented in Tables 32 and 33 and graphed in Figure 23.

1,1,1-Trichloroethane (TCE 1,1) --

Only two doses of TCE 1,1 were tested, 100 and 1000 mg/kg. Table 34 indicates that the lower dose causes both an increase in tritiated thymidine pool size and a decrease in testicular DNA synthesis to 60% of control values. Neither effect is statistically significant (Table 35). The higher dose produces even a greater inhibition of testicular DNA synthesis (Figure 24). As a result, it is concluded that the decrease in DNA synthesis caused by the lower dose is real, and will be called "biologically significant."

It is the large variances in the results obtained from successive repetitions at the given dose level which have caused this discrepancy between "statistical significance" and "biological significance." Biological significance should not be excluded from the presentation of elicited effects, and the reason for the effects not being statistically significant should be determined and eliminated, if possible. For example, the effect may be there, with very little variance about the quantitated observations, but may not be great enough at the given dose to be statistically significant. These results would imply that higher doses of the compound need to be tested, and should not be interpreted to mean that the compound elicits no effects at all.

1,2-Dichloroethane (DCE 1,2) --

Doses of DCE 1,2 ranging from 4.9 to 250 mg/kg were tested for their effects upon testicular DNA synthesis. No effects upon either total testicular counts or tritiated thymidine pool size were elicited. At the highest dose administered, 250 mg/kg, a statistically significant decrease in testicular DNA synthesis was observed. This dose causes an approximate 50% inhibition of testicular DNA synthesis. These data are presented in Tables 36 and 37 and graphed in Figure 25.

Compound Comparisons

In order to compare the relative capabilities of the five halocarbons to inhibit mouse testicular DNA synthesis, the data from all studies was compiled and graphed (Figures 26 and 27). In both figures, the doses administered have been plotted on a millimolar basis in order to compensate for the different molecular weights of the compounds. A list of the mg/kg doses and their equivalent millimolar/kg doses is presented in Table 38. In Figure 27, the log of the millimolar dose administered was plotted to compensate for the exponential nature of dose-response curves derived from

TABLE 32. EFFECTS OF TCE 1,2 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	DPMH		DPMS1		DPMSF		DNAMICG	
(mg/kg)	Mean	% ± SEM^N	Mean	% ± SEM^N	Mean	% ± SEM^N	Mean	% ± SEM^N
0			12542765	100 ± 15^6	3969789	100 ± 7^6	6244	100 ± 5^6
3.8			13160845	105 ± 15^5	3439114	87 ± 11^5	6268	100 ± 3^5
3.8			10135466	81 ± 10^4	3619753	91 ± 1^4	5793	93 ± 9^4
0	13373135	100 ± 15^7	10929435	100 ± 5^7	3344964	100 ± 8^7	5334	100 ± 5^7
75	16840399	126 ± 20^7	15323801	140 ± 14^7	3411469	102 ± 14^7	4952	93 ± 8^7
150	13907316	104 ± 9^7	13632514	125 ± 11^7	3270235	98 ± 8^7	5488	103 ± 6^7
300	15544588	116 ± 10^6	18108974	166 ± 19^6	1045819	31 ± 6^6	4828	91 ± 7^6
600	23237020	174 ± 15^7	34590581	316 ± 25^7	203938	6 ± 1^6	5530	104 ± 6^7
0			10715724	100 ± 11^8	4723595	100 ± 8^8	4631	100 ± 6^8
300			12405577	116 ± 20^8	3226735	68 ± 9^8	5569	120 ± 8^8
600			25165753	235 ± 12^8	674297	14 ± 3^8	5361	116 ± 11^8
800			26448858	247 ± 29^8	25471	1 ± 0^5	5649	122 ± 7^8
1200			24005194	224 ± 36^8	27364	1 ± 0^7	5587	121 ± 5^8

TABLE 33. STATISTICAL ANALYSIS OF EFFECTS OF TCE 1,2 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.65		.75	A	.26		.49	A	.89		.47	A	.28		.55	A	.32		.62	A
38				A				A				A				A				A
38				A				A				A				A				A
0	.00		.04	B	.00		.00	C	.00		.00	A	.61		.49	A	.59		.50	A
75				AB				B				A				A				A
150				B				BC				A				A				A
300				AB		+		B		+		B				A				A
600		+		A		+		A		+		C				A				A
0	.12		.07	B	.00		.00	B	.00		.00	A	.04		.12	B	.08		.22	A
300				B				B				A				A				A
600				A		+		A		+		B				AB				A
800				AB		+		A		+		C				A				A
1200				AB		+		A		+		C				A				A

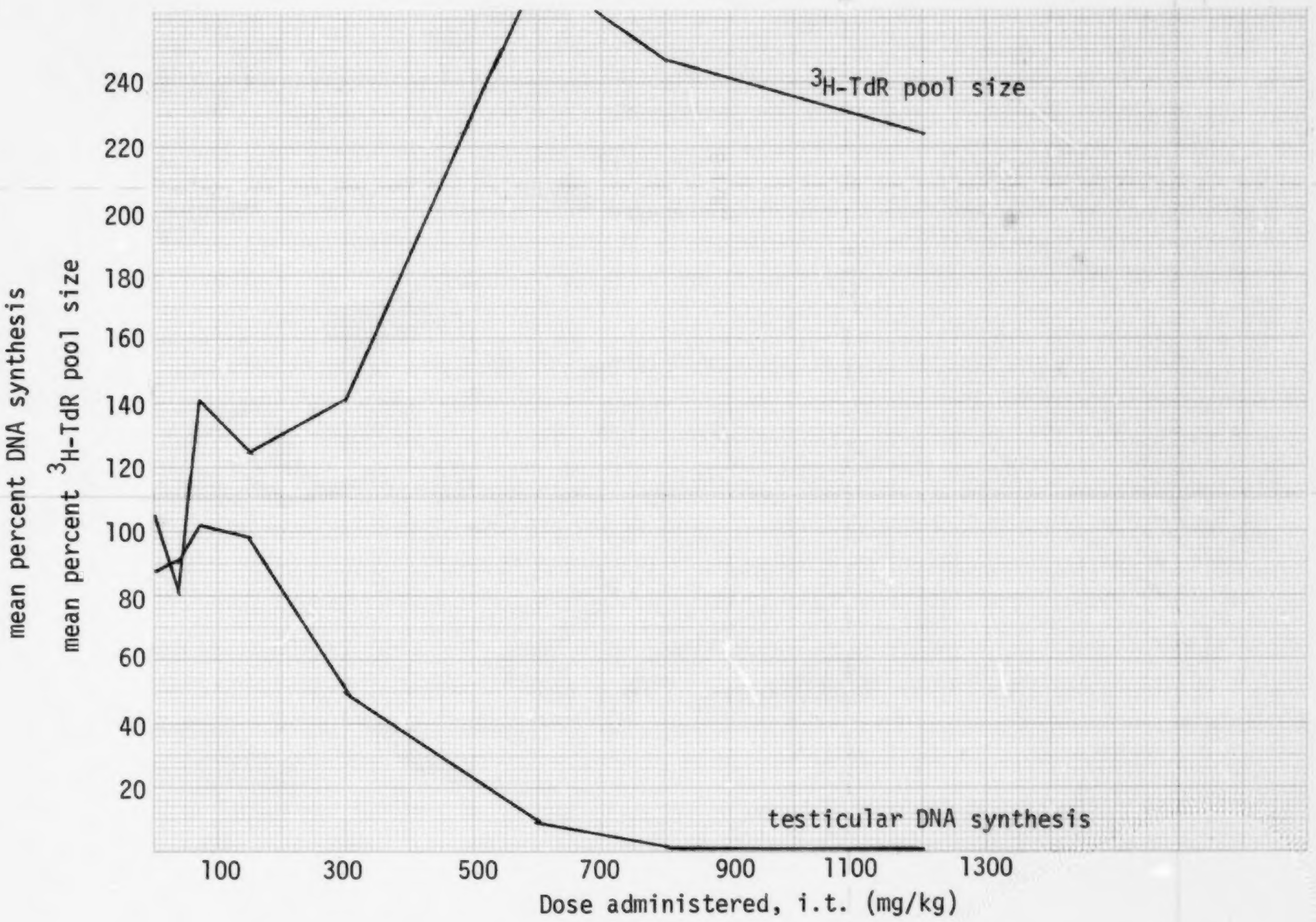

Figure 23. Effects of TCE 1,2 upon mouse testicular DNA synthesis.

TABLE 34
EFFECTS OF TCE 1,1 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	DPMS1		DPMSF		DNAMICG	
(mg/kg)	Mean	$\% \pm \mathrm{SEM}^N$	Mean	$\% \pm \mathrm{SEM}^N$	Mean	$\% \pm \mathrm{SEM}^N$
0	10758439	100 ± 11^6	3425973	100 ± 14^6	5009	100 ± 5^6
100	19561906	182 ± 45^5	2071357	60 ± 12^5	6100	122 ± 7^5
1000	14339143	133 ± 23^5	922262	27 ± 13^4	4986	100 ± 17^5

TABLE 35. STATISTICAL ANALYSIS OF EFFECTS OF TCE 1,1 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.73		.14	A	.83		.15	A	.00		.01	A	.37		.35	A	.54		.27	A
100				A				A				A				A				A
1000				A				A		+		B				A				A

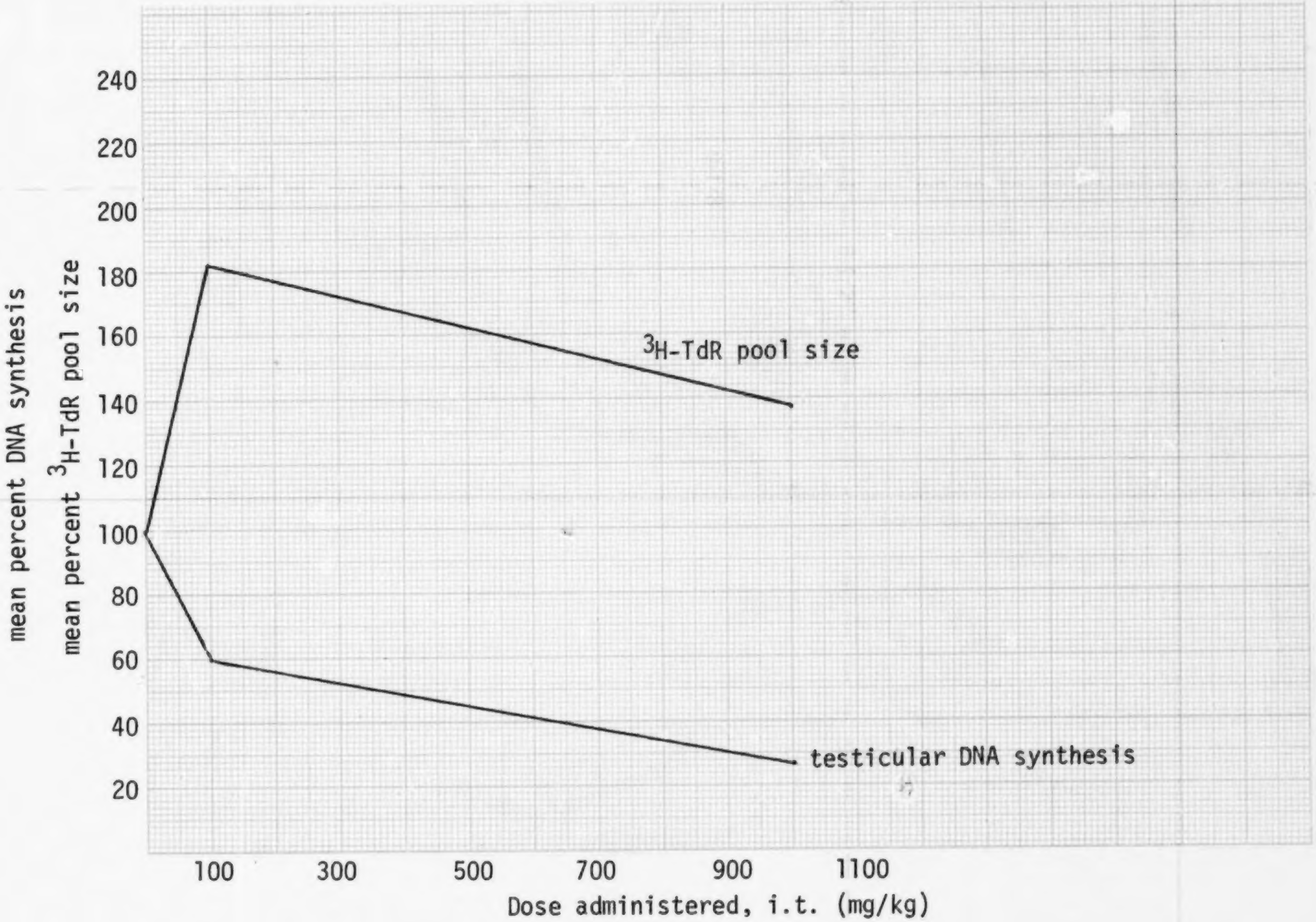

Figure 24. Effects of TCE 1,1 upon mouse testicular DNA synthesis.

TABLE 36. EFFECT OF DCE 1, 2 UPON MOUSE TESTICULAR DNA SYNTHESIS

DOSE (mg/kg)	DPMH Mean	DPMH % ± SEM^{N}	DPMS1 Mean	DPMS1 % ± SEM^{N}	DPMSF Mean	DPMSF % ± SEM^{N}	DNAMICG Mean	DNAMICG % ± SEM^{N}
0			14711934	100 ± 32^{6}	4220283	100 ± 16^{6}	5568	100 ± 6^{6}
4.9			12838412	87 ± 17^{5}	4456587	106 ± 20^{5}	4371	79 ± 4^{5}
49			15324057	104 ± 8^{5}	3737688	89 ± 11^{5}	4769	86 ± 6^{5}
0	22081439	100 ± 17^{7}	13828287	100 ± 8^{4}	4465641	100 ± 7^{7}	5742	100 ± 7^{7}
10	21781426	99 ± 13^{7}	12705499	92 ± 6^{7}	4321897	97 ± 10^{7}	5109	89 ± 5^{7}
50	24342643	110 ± 18^{6}	16560783	120 ± 30^{6}	3510700	79 ± 8^{6}	5584	97 ± 9^{6}
100	22331112	101 ± 19^{7}	14698562	106 ± 10^{7}	4169198	93 ± 5^{7}	5304	92 ± 11^{7}
250	22788549	103 ± 16^{7}	15431906	112 ± 23^{7}	2112359	47 ± 7^{7}	4755	83 ± 8^{7}

TABLE 37. STATISTICAL ANALYSIS OF EFFECTS OF DCE 1,2 UPON MOUSE TESTICULAR DNA SYNTHESIS

Dose	LDPMH				LDPMS1				LDPMSF				LDNAMICG				DNAMICG			
(mg/kg)	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn	LR	Dt	A	Dn
0	.19		.20	A	.39		.70	A	.59		.85	A	.47		.03	A	.44		.03	A
4.9				A				A				A		+		B		+		B
49				A				A				A				AB				AB
0	1.00		.99	A	.81		.94	A	.00		.00	A	.22		.71	A	.20		.62	A
10				A				A				A				A				A
50				A				A				A				A				A
100				A				A				A				A				A
250				A				A		+		B				A				A

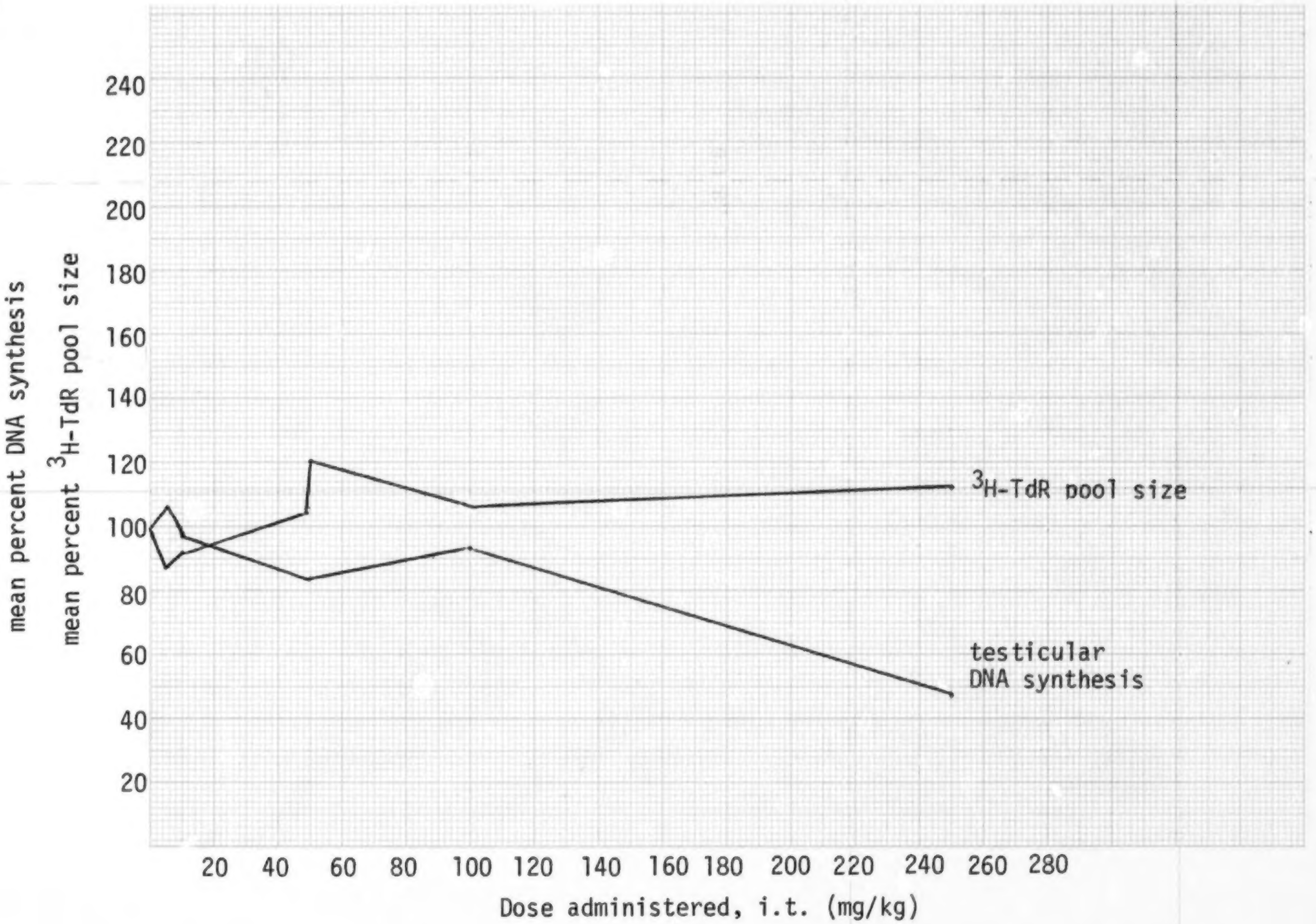

Figure 25. Effects of DCE 1,2 upon mouse testicular DNA synthesis.

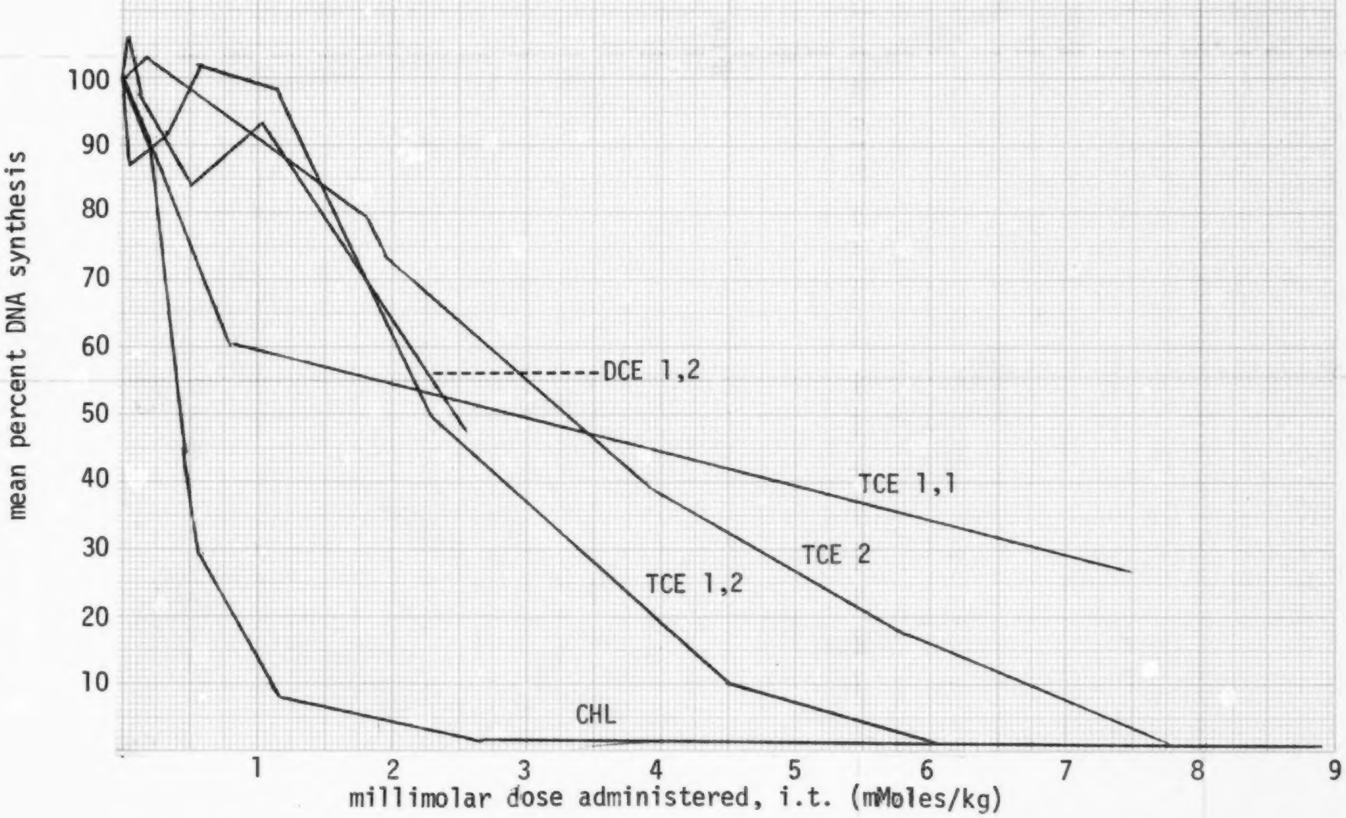

Figure 26. Comparisons of the effects of CHL; DCE 1,2; TCE 1,1; TCE 1,2; and TCE 2 upon testicular DNA synthesis, viewed on a millimolar dose basis.

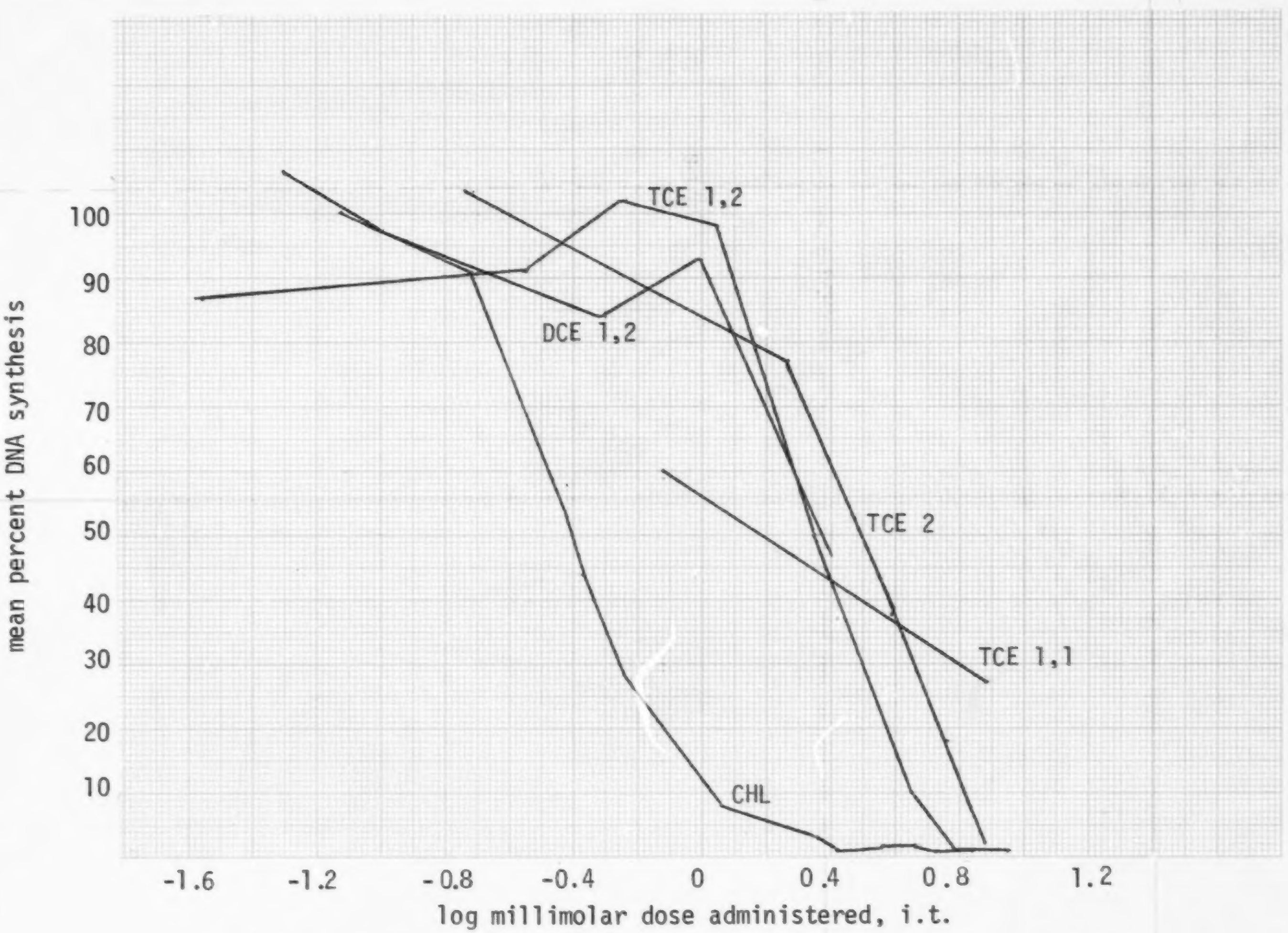

Figure 27. Comparisons of the effects of CHL; DCE 1,2; TCE 1,1; TCE 1,2; and TCE 2 upon testicular DNA synthesis, viewed on a log(millimolar dose) basis.

TABLE 38
MG/KG DOSES AND EQUIVALENT MILLIMOLAR/KG DOSES FOR THE HALOCARBONS STUDIED

Compound	Dose (mg/kg)	Equivalent Millimolar Dose/kg
CHL	10	.076
M.W. = 165.42	25	.190
	50	.381
	56	.426
	75	.571
	150	1.14
	300	2.28
	350	2.66
	474	3.61
	600	4.57
	700	5.33
	900	6.85
TCE 2	24	.183
M.W. = 131.39	240	1.83
	250	1.90
	500	3.81
	750	5.71
	1000	7.61
TCE 1,2	3.8	.028
M.W. = 133.40	38	.285
	75	.562
	150	1.12
	300	2.25
	600	4.50
	800	6.00
	1200	9.00
TCE 1,1	100	.75
M.W. = 133.40	1000	7.50
DCE 1,2	4.9	.05
M.W. = 98.96	10	.101
	49	.495
	50	.505
	100	1.01
	250	2.53

biological systems. In viewing Figure 27, it appears that the dose-response curves of TCE 2; TCE 1,2; DCE 1,2; and CHL may have equivalent slopes. This implies that these compounds may be causing their effects through the same mechanism. The curves for CHL and TCE 1,1, however, lie apart from the others. In Table 39, the minimum doses producing significant decreases in testicular DNA synthesis (Dunnett's t-test) have been listed. These data confirm the observations that the dose-response curves for DCE 1,2; TCE 2; and TCE 1,2 are very similar. The minimum dose of CHL producing a significant effect (0.83 mMoles/kg), however, is much lower than those of the other compounds. One possible explanation for this lies in the similarities and differences between the structures of the five compounds.

The structures are illustrated in Figure 1. Comparisons reveal that only CHL has an aldehyde group. Perhaps this is the basis for its increased ability to inhibit testicular DNA synthesis. TCE 1,1, on the other hand, also differs significantly from the other compounds. Because all three of its chlorine atoms are bound to the same carbon atom, it is the most polar of the compounds. Perhaps this is the basis for a dose-response that is different from the others.

In summary, it appears that the dose-response curves for inhibition of testicular DNA synthesis by four of the five compounds, TCE 2; TCE 1,2; DCE 1,2; and CHL have similar slopes, implying that all four compounds may cause their effects through the same mechanism. The leftward shift of the CHL curve indicates that the mechanism by which this effect is elicited is more sensitive to CHL than to the other compounds. The curve for TCE 1,1 appears to lie separately from the others which is perhaps explained by its greater polarity in comparison to the other compounds. Because only two dose levels were used in the testing of TCE 1,1, more experiments would need to be conducted before any firm conclusions may be drawn concerning the relative shift of the TCE 1,1 dose-response curve.

TABLE 39. COMPOUND COMPARISONS - INHIBITION OF MOUSE TESTICULAR DNA SYNTHESIS

COMPOUND (Abbrev.)	DOSE* (mMoles/kg)	DOSE* (mg/kg)	PERCENT TESTICULAR DNA SYNTHESIS†	HIGHEST DOSE NOT PRODUCING SIGNIFICANT EFFECTS (mg/kg)
CHL	0.38	50	53% ± 4%§	25
DCE 1,2	2.53	250	47% ± 7%	100
TCE 2	3.81	500	40% ± 7%	250
TCE 1,2	2.25	300	31% ± 6%	150
TCE 1,1	7.50	1000	27% ± 13%	100

* The lowest dose producing a statistically significant decrease in testicular DNA synthesis; Dunnett's t-test, $p \leq 0.05$.

† Expressed as a percent of the vehicle control response, defined as 100%.

§ Standard error of the mean.

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